CHARACTERIZATION OF LOCALLY ISOLATED MICROALGAE PARACHLORELLA KESSLERI FOR CO_2 FIXATION AND BIOFUEL PRODUCTION

BY

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This work is dedicated to my beloved parents, sister, wife and children.

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ABSTRACT

Full Name : Abdulwadod Wahid Mohammed

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Global warming is a major threat to the living organisms and environmental sustainability due to the increase in greenhouse gases (GHGs) emissions, especially CO_2 produced by industries and transportations. Technologies have thus been developed for enhanced biological carbon fixation through microalgae. In addition, utilizing microalgae for biofuel production has attracted enormous research interests in recent years, mainly because of their high capability to convert CO_2 photosynthetically into potential biofuel biomass and high value biochemicals. Accordingly, the current research is focused to optimize the photoautotrophic cultivation conditions of a locally isolated indigenous microalgal strain namely *Parachlorella kessleri and* analyze its capability to capture CO_2 then further produce biofuels under optimized conditions. Different culture conditions were optimized, nitrogen concentration and NP ratio under different light intensities. Furthermore, nitrogen starvation has been investigated and CO_2 fixation rate has been studied with different light intensities. Increasing the light intensity resulted in high microalgal growth, productivity, CO_2 fixation capability and lipid accumulation. The locally isolated *Parachlorella kessleri* strain shows high potential for enhanced microalgal CO_2 fixation, and the possibility of coupling wastewater treatment with microalgal growth to produce biofuels or high valuable chemicals.

ملخص الرسالة

الاسم الكامل : عبدالودود وحيد محمد

عنوان الرسالة : توصيف الطحلب الدقيق والمعزول محلياً باراكلوريلا كيسلاري لتثبيت غاز ثاني أكسيد الكربون وإنتاج الوقود الحيوي

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يشكل الاحتباس الحراري تهديدًا كبيرًا للكائنات الحية والاستدامة البيئية بسبب زيادة انبعاثات الغازات الدفيئة خاصةً ثاني أكسيد الكربون الناتج عن المصانع ووسائل المواصلات المستهلكة للوقود الأحفوري. لقد تم تطوير تقنيات حديثة لتعزيز تثبيت الكربون حيوياً عن طريق الطحالب الدقيقة، بالإضافة إلى أن استخدام الطحالب المجهرية الدقيقة لإنتاج الوقود الحيوي قد اجتذب اهتمامات بحثية هائلة في السنوات والعقود الأخيرة وذلك يرجع أساسًا إلى قدرتها العالية على تحويل ثاني أكسيد الكربون من خلال عملية البناء الضوئي إلى كتلة للوقود الحيوي والمواد الحيوكيميائية ذات القيمة العالية. بناءً على ذلك يركز بحثنا الحالي على تحسين ظروف ومقومات زراعة الطحالب الدقيقة ذاتية التغذية والمعزولة محلياً والمعروفة ب*Parachlorella kessleri* والقيام أيضاً بتحليل قدرتها على تثبيت ثاني أكسيد الكربون لإنتاج الوقود الحيوي في ظل الظروف التي تم تحسينها مسبقاً والتي تتمثل بإيجاد التركيز الأمثل للنيتروجين ونسبة النيتروجين إلى الفسفور تحت مستويات شدة ضوء مختلفة. وكما تمت دراسة تأثير الإجهاد النيتروجيني الغذائي على نمو الطحالب الدقيقة ومعدل تثبيت ثاني أكسيد الكربون تحت مستويات شدة ضوء مختلفة أيضاً. لوحظ أن زيادة شدة الضوء يساهم في زيادة معدل النمو وإنتاجية الطحالب المجهرية الدقيقة وقدرتها على تثبيت ثاني أكسيد الكربون وتجميع الدهون. تظهر سلالة الطحالب الدقيقة *Parachlorella kessleri* المعزولة محليًا إمكانات عالية لتثبيت ثاني أكسيد الكربون وإنتاج الدهون التي يتم استخراجها وتحويلها إلى وقود حيوي. وأخيراً من الممكن ربط معالجة مياه الصرف الصحي مع زيادة نمو الطحالب الدقيقة وذلك لإنتاج الوقود الحيوي أو المواد الكيميائية ذات القيمة العالية.

CHAPTER 1

INTRODUCTION

1.1 Global Warming

Global warming is one of the major threats to living organisms in this planet. It is defined as the rising of the average Earth's surface temperature due to the buildup of greenhouse gases (GHGs) emissions and concentrations in the atmosphere, especially CO_2 emission, that primarily trap the heat within the atmosphere. An international cooperation has been carried out to reduce the emissions of GHGs. Many nations agreed to restrain the rising global average temperature by a ceiling of lower than 2 °C in order to avoid serious consequences of the global warming. But this international agreement is accompanying with extensive technological, institutional and economical challenges. Nowadays, the world tends to use more clean, efficient and sustainable energy to reduce the emissions of GHGs rather than relying on burning coal and fossil fuels.

Carbon emissions from global fossil fuels has tremendously increased later 1900 AD. CO_2 emissions have increased since 1970 by approximately 90% as shown in Figure 1. Combustion units, industrial processes, agriculture and deforestation are the major causes of the total GHGs emissions increase from 1970 to 2011. Saudi Arabia is one of the largest oil producer and it ranks among the top ten nations based on 2015 fossil fuel CO_2 emissions with 506.6 million metric tons of carbon that represents 253% percent higher compared to

CO_2 emissions in 1990 [1], while its contribution still is increasing in recent years. Thus, development in CO_2 capture, reduction and utilization will be mandatory to maintain a sustainable environment and economic expansion.

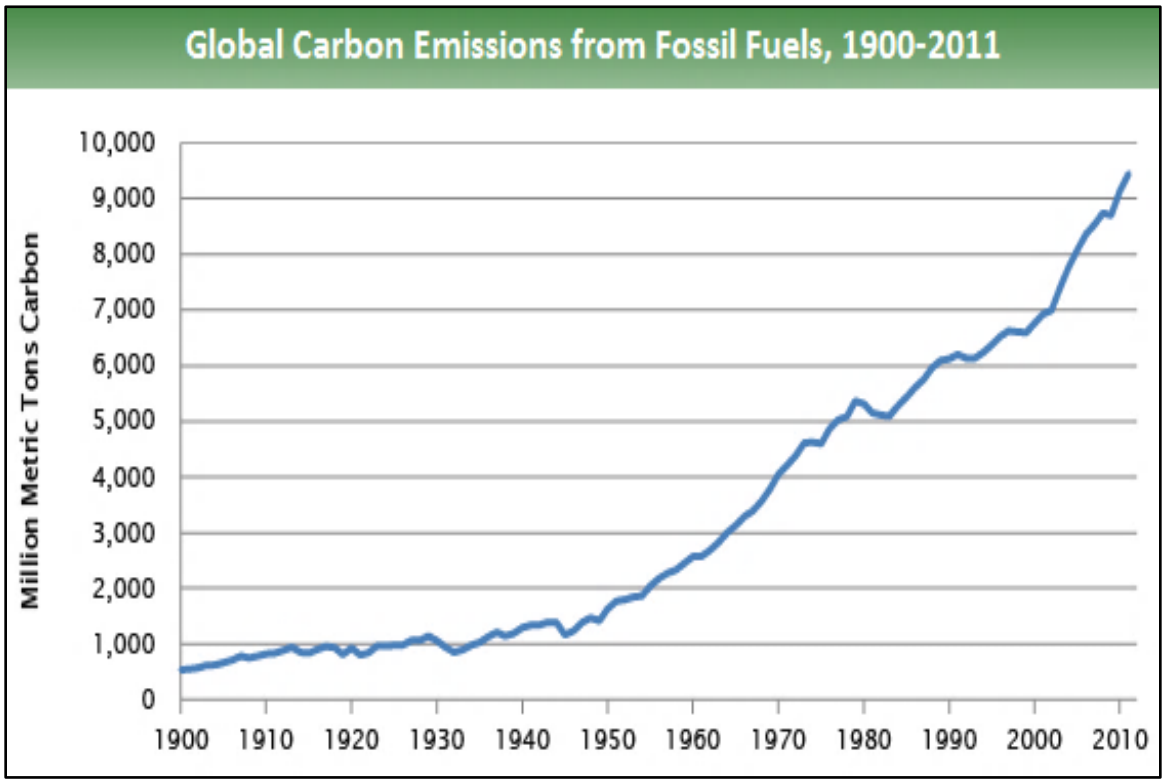


Figure 1. Global, Regional, and National Fossil Fuel CO_2 Emissions [2].

1.2 CO_2 Emission and Mitigation

Global temperature and GHGs emissions are still increasing in atmosphere while CO_2 concentration has reached 406 ppm and it is predicted to reach 450 ppm by 2035 [3]. Global warming has several deleterious effects such as low food productivity, unexpected climate variation, increase water acidity and ocean levels. As evaluated by EIA, the highest contributors of CO_2 emissions are the power generation, industrial and transportation sectors [4] as can be seen in Figure 2.

Mitigation is a human intervention to diminish the sources of GHGs. Carbon capture and utilization (CCU) is an expeditious advanced technology to mitigate the anthropogenic CO_2 emissions effect. CCU is relying on CO_2 capture from a point source emitter and then converting that CO_2 to valuable and innovative new products through chemical or biological techniques [5,6]. The CO_2 capture process can be done by three main methods [7,8]:

Pre-combustion: Involves the syngas production via gasification or reforming with presence of air or oxygen, and separation of CO_2 and hydrogen.

Oxyfuel combustion: Includes burning of hydrocarbon or biomass fuels in pure oxygen instead of air to reduce N_2 content and produce highly concentrated CO_2 and water.

Post-combustion: Take in the capturing of CO_2 from fossil fuel combustion flue gases primarily through absorption, cryogenic distillation or membranes.

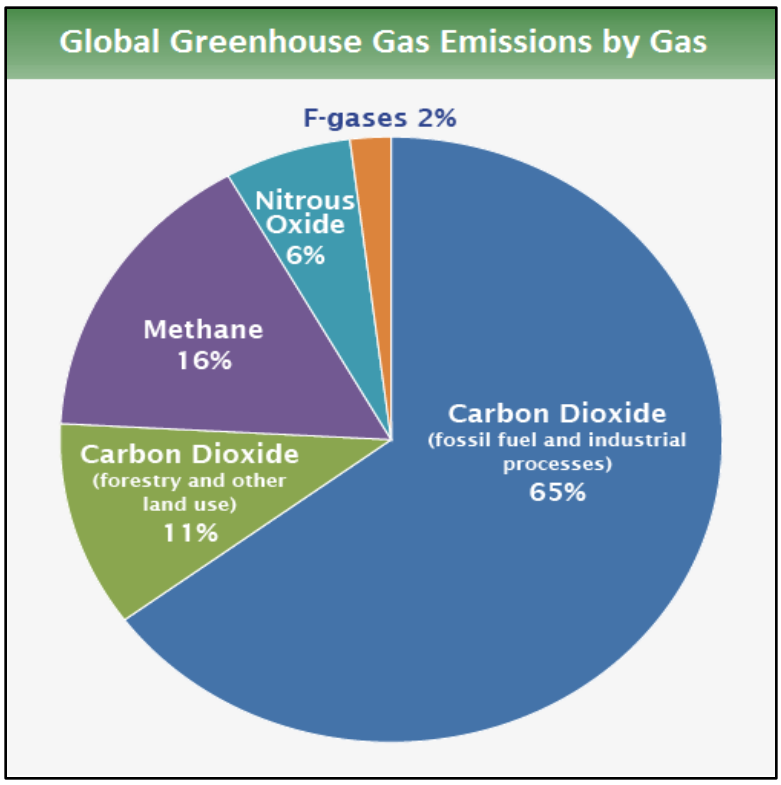


Figure 2. Global Emission Data [9].

Several techniques are proposed for CCU like bio-fixation of CO_2 or direct catalytic conversion. Biological systems are more competent in utilizing CO_2 than chemical conversion techniques. CO_2 sequestration can be achieved through many natural processes such as forestation, ocean fertilization and microbes [10]. Microalgae-based CO_2 bio-fixation to produce biofertilizers, bio-fuels or high value chemicals from resultant biomass is an attractive and promising technique. Many researchers have investigated microalgae as an alternative approach for CO_2 capture [11].

1.3 Microalgae

Microalgae are unicellular and multicellular photosynthetic species that exist individually, in chains or groups, and can be found in freshwater or marine systems. Their biochemical diversity involves production of a wide collection of commercially valuable lipids, carbohydrates and proteins [12]. Microalgae convert CO_2 into chemical compounds using sun light through photosynthesis then store a portion of the resulted energy as reduced carbon in photoautotrophs. Biofuel feedstock generations are divided into three categories, which are food yields (first generation), non-food yields (second generation) and microalgae (third generation). The biofuel production cost for large scale commercial usage remains a main obstacle because of the high feed cost of food oils. Moreover, first and second generations biofuel feedstocks are inefficient and unsustainable for long term and high yield. Alternatively, third generation biofuel feedstock have distinguished as an excellent alternative lipid source for biofuel production owing to the high productivity, growth rates and high photosynthetic efficiency of microalgae for biomass production in contrast with traditional crops. Furthermore, microalgae can be cultivated easily due to their fast reproduction compared to many other types of plants, as a result they are producing a higher oil yield and biomass productivity as shown in Table 1. High oil content in microalgae have the possibility to produce around 25 times higher yield than the conventional palm oil. Due to these characteristics microalgae has been chosen preferably as an excellent source of biofuels production as compared to the conventional oil crops [13,14].

Table 1. Comparison of Microalgae with Other Biofuel Feedstocks [13].

Plant Source	Oil Weight %	Oil Yield [L/ha/year]	Land Use [m^2-year/kg]	Biofuel Productivity [kg/ha/year]
Microalgae (High)	70	136,900	0.1	121,104
Microalgae (Medium.)	50	97,800	0.1	86,515
Microalgae (Low)	30	58,700	0.2	51,927
Palm Oil	36	5,366	2	4,747
Castor	48	1,307	9	1,156
Sunflower	40	1,070	11	946
Canola	41	974	12	862
Camelina	42	915	12	809
Jatropha	28	741	15	656
Soybean	18	636	18	562
Hemp	33	363	31	321
Corn	44	172	66	152

Microalgae have several advantages as follows [15–21] , where Microalgae:

- Can produce useful by-products like biopolymers carbohydrates to be used as biofertilizers.
- Can grow under wide range of CO_2 concentrations (1-60%)
- Can be used in CO_2 bio-fixation (1.0 kg of dry biomass needs 1.8 kg of CO_2).
- Cultivation can be integrated with wastewater treatment through the removal of phosphorus, nitrogen, and heavy metals.
- Have low harvesting and transportation cost.

- Require small land area and can be grown in different environments.
- Can double their biomass in daily basis with high growth rates.
- Can produce a higher yield per hectare with better quality and improved ecological performance.
- Have a high photosynthetic efficiency.

Notwithstanding many features, the cost of microalgae CO_2 fixation is still higher compared to other carbon capture and storage (CCS) technologies. Nevertheless, the concept of converting microalgae biomass into valuable chemicals is anticipated to offset the CO_2 capture cost. Also, the lack of large scale experimental data under real environmental conditions must be investigated. Microalgal strains that can tolerate high temperatures, CO_2 concentrations and growth in acidic environment are desired [22]. Many challenges need to be overcome for maximum production of high value chemicals which involve combination of cultivation technologies and development of potential microalgae species [23]. Based on current global assessment, Saudi Arabia has a conducive environment for microalgae cultivation and biomass production under relatively high temperatures and the availability of large uncultivatable areas across the kingdom. Microalgae biomass can further be utilized for value added chemicals [24].

1.4 Research Objectives

The main objectives pursued for this research are the following:

1. Isolation To optimize the growth medium for locally isolated indigenous microalgal strain *Parachlorella kessleri*.
2. To study the growth kinetics of isolated microalgae under different nutrient concentrations and light intensities.
3. To evaluate the microalgal CO_2 fixation capability under optimized conditions of nutrient concentrations.
4. To analyze the biomass compositions and evaluate the its potential for biodiesel production.

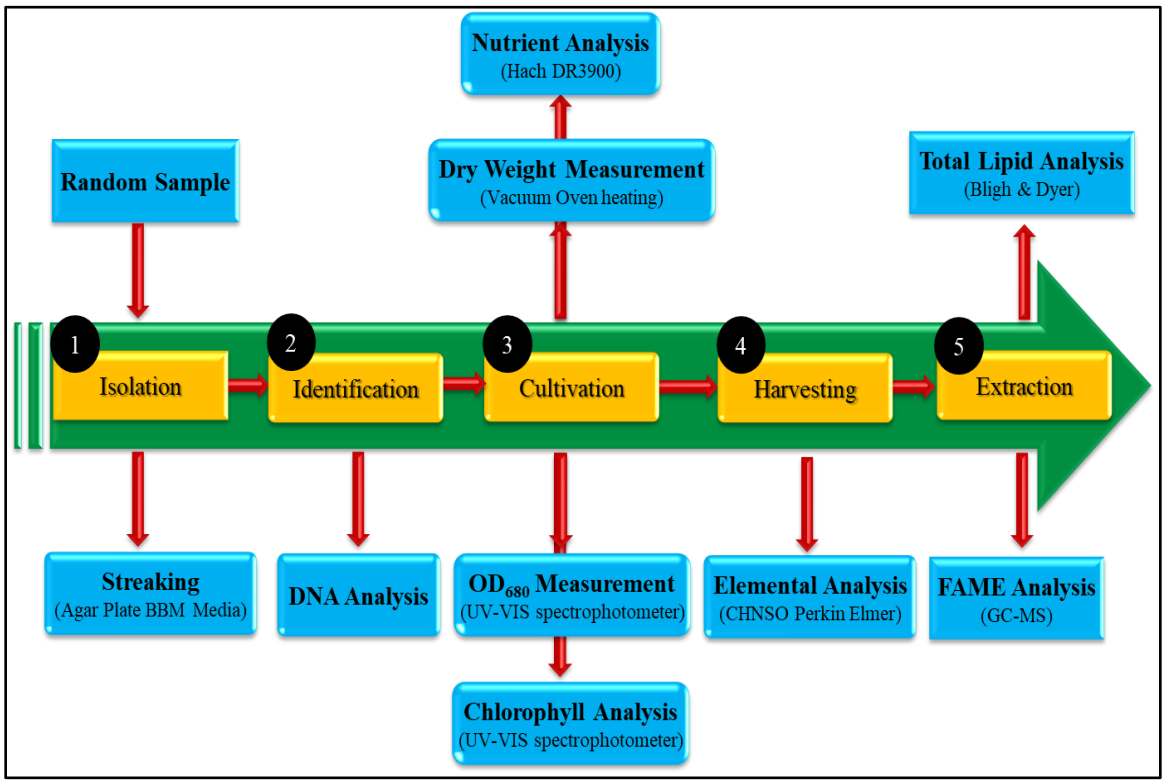


Figure 3. Research Process Scheme.

CHAPTER 2

LITERATURE REVIEW

2.1 CO_2 Capture

Mitigation is an important scientific approach used to diminish the sources of GHGs. Carbon capture and utilization (CCU) is an advanced technology to mitigate the anthropogenic CO_2 emissions effect. CO_2 capture from a point source emitter and then converting that CO_2 to valuable and innovative new products through chemical or biological techniques [5,6].

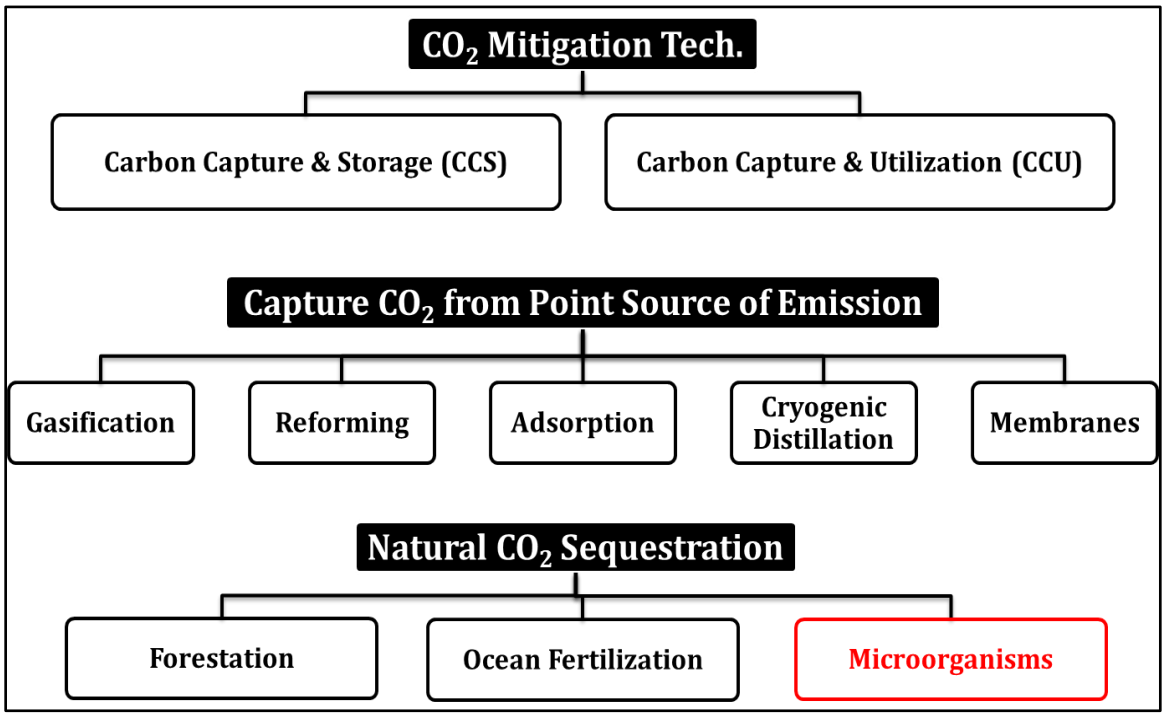


Figure 4. CO_2 Mitigation Technologies.

2.2 Open and Closed Cultivation System

Microalgae cultivation systems are classified as open and closed systems [25]. Open cultivation systems are subjected to various weather conditions. Seasonal weather fluctuations possibly will affect the temperature and light intensity. Extensive amounts of water evaporate resulting in a reduction of working volume of media. Moreover, the water level had better be kept at low levels to permit sunlight to reach all the microalgal culture. In an open system, contaminants or unwanted microorganisms, such as bacteria, can compete with the microalgae for nourishment which may limit the microalgal growth. Therefore, in open systems the microalgae possibly will grow under detrimental conditions. Additionally, the CO_2 available naturally in the atmosphere represent around 0.03% which limits the CO_2 required for photosynthesis process. Furthermore, large areas required for open cultivation is another critical issue for microalgae cultivation [26]. Cultivation in a closed system can provide proper controlling for the microalgae growth conditions and environment especially for single colony cultivation [18]. Closed systems increase the light accessibility and provide well-mixing process to improve gas exchange and optimum cell growth. Closed systems can be operated under different weather conditions by using several microalgae strains. Also, it can minimize the loss of CO_2 through regulation of air-CO_2 mixing and flow rates. In addition, closed systems are perfect for contamination-free cultivation. Moreover, temperature can be controlled or adjusted to the desired values [27,28]. However, closed systems require high installation and operation costs. Also, it can be fouled by microalgae when they grow and stick on the wall of the reactor, which lower the accessibility of light and consequently lower the biomass production [29].

2.3 Growth Parameters

Scientists are studying different parameters that affect the microalgae growth and biomass production linked with CO_2 capture and mitigation. Several experiments have been done using different strains of microalgae that have been cultivated in different media. The adjustable parameters involved in maximizing algal and lipid productivity experiments are nutrient concentration, temperature, light intensity, CO_2 concentration, diurnal / nocturnal cycles and gas flow rate. Further review will discuss the effect of some parameters of interest on biomass or lipid productivity. Figure 5 shows several parameters that affect the cultivation process of microalgae [12].

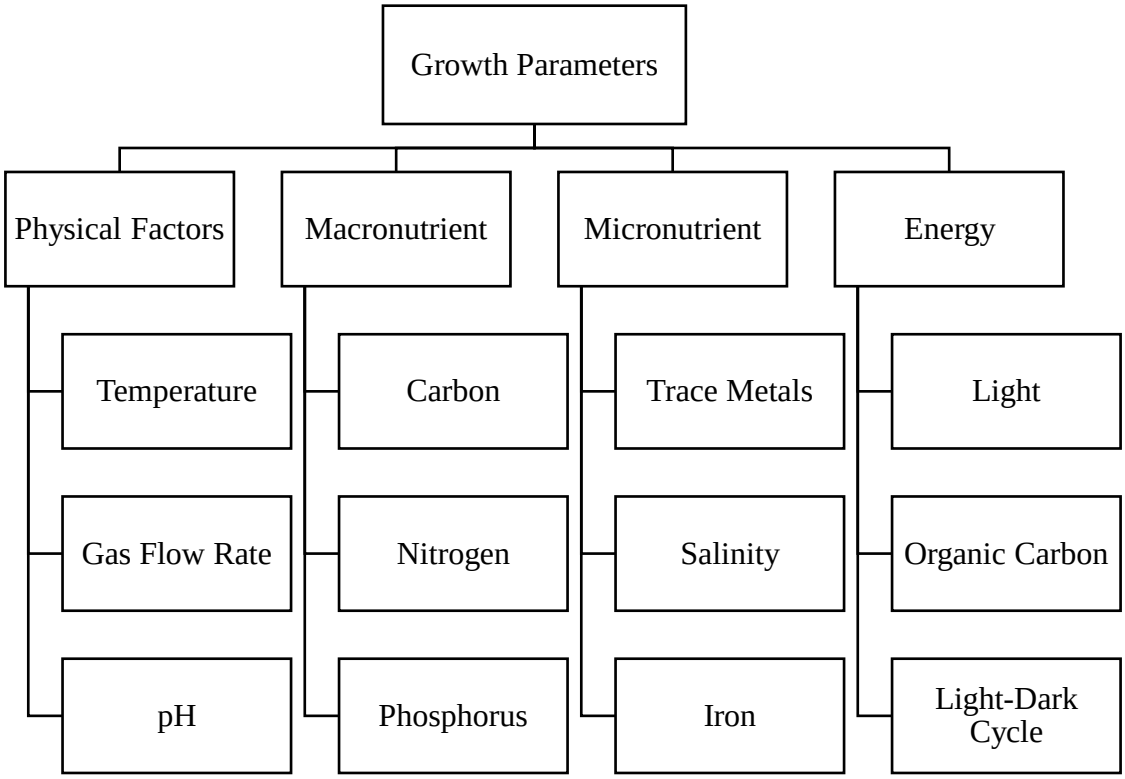


Figure 5. Growth Parameters Schematic of Microalgae.

2.4 Nutritional Modes

Algae nourishment altered around two main modes of nutrition which are autotrophy and heterotrophy [12].

2.4.1 Autotrophic Culture

Autotrophic organisms acquire their energy throughout the absorption of light for CO_2 reduction by substrate oxidation. Cultures that only need inorganic minerals in the presence of visible light for growth are known as photoautotrophs. Photoautotrophic cultures can produce hydrocarbons, proteins, carbohydrates and lipids through photosynthesis [21]. Some algae cultures can attain more energy by further oxidation of inorganic compounds which refers to them as chemoautotrophs [12].

2.4.2 Heterotrophic Culture

Heterotrophic organisms acquire their energy and food from the organic substrates. Hence, light is not demanded for the growth of cells [30]. High lipid productivity noticed under heterotrophic cultures, around twenty folds, compared to photoautotrophic cultivation depending on algal strain type, growth parameters and culturing mediums [31]. For example, Mendes et al. [32] studied the Docosahexaenoic acid (DHA) production using anaerobic growth of the strain *Crypthecodinium cohnii*. Some cultures are known as photoheterotrophs which can acquire the energy though visible light to utilize organic compounds as food for growth. Photoheterotrophic cultures are mainly used for hydrogen production [33,34]. Cultures that can oxidize organic substrates for energy are known as

chemoheterotrophs, while cultures that digest food through absorption of nutrients particles into vesicles are known as phagocytotic algae [12].

2.4.3 Mixotrophic Culture

Autotrophy and heterotrophy can both present depending on the presence of visible light and availability of carbon source [14]. For example, Lee et al. [35] studied the growth of *Chlorella sorokiniana* and reported the heterotrophic growth during night and mixotrophic growth during the day. In heterotrophic growth, *Chlorella sorokiniana* is utilizing glucose, while in the day time it is depending on both CO_2 and glucose. Table 2 summarizes the nutritional modes [36]:

Table 2. Summary of Nutritional Modes.

Nutritional Mode	Nutrients Source	Energy Source
Autotrophic Mode	Inorganic Substrate	Light
Heterotrophic Mode	Organic Substrate	Organic Substrate
Mixotrophic Mode	Inorganic & Organic Substrate	Light & Organic Substrate

2.5 Nitrogen Concentration

Photoautotrophic cultivation requires three essential nutrients for cell growth and life sustainability which are nitrogen, carbon and phosphorus [12]. Nitrogen is a vital inorganic nutrient required for biomass production which represents around 1 to 10% of the cell biomass depending on the availability among different species. Nitrogen limitation responses can typically be organic carbon compounds accumulation and discoloration [37]. Nitrogen can be supplied as urea, ammonia or nitrate as reported by Kaplan et al. [38] and these three forms of nitrogen have resulted in similar growth rates. Ammonia can be used only as a source of nitrogen, but the pH level could decrease extensively due to H^+ ions released throughout the active growth process. However, supplying only nitrate as source of nitrogen will increase the pH level. The key factor in microalgal biotechnology, that aimed to attain high yields, is to ensure satisfactory supply of the essential nutrients. Excess supply of nitrogen in culture media could be toxic. Instead, nitrogen should be supplied in limiting concentrations.

Several factors could affect the microalgae metabolism extensively including physicochemical stress factors. Such type of stress was used in the laboratory to produce lipid from microalgae in pilot-scale. Another common stress factor used for improving lipid production is nitrate [39]. Lipid productivity was improved with increasing the concentration of nitrate in media of *Scenedesmus sp.* and *Auxenochlorella pyrenoidosa* [40,41]. An opposite approach was observed in *Chlorella vulgaris* and *Auxenochlorella protothecoides* which enhanced the lipid production was higher at lower nitrate concentrations [42,43]. Recent studies are focused on microalgal cultivation under nitrogen

stress condition to produce high lipid and biomass productivity. In this regard, two nitrogen stress approaches have been used to enhance the production of lipids in numerous strains of microalgae. The first strategy is called single stage cultivation where the culture medium involves certain concentration of nitrogen. As a result, the nutrient starts depleting as the culture grows until the starved conditions is accomplished [44]. The second strategy is called two stage nitrogen depletion where the microalgal cells are cultivated in nitrogen rich conditions to intensify the growth of cell biomass and then shifted into nitrogen starved medium for improving the production of lipids. Two stage nitrogen depletion have been studied as shown in Table 3. Optimal nitrogen concentration was recorded in *Nannochloropsis oculata* at 47.6 ppm and in *Scenedesmus obtusus* at 49.2 ppm [45,46]. Also, optimal nitrogen concentration was reported in *Chlorella vulgaris* at 43.4, 55.8 , 41.3 ppm, respectively [47–49]. Similarly, optimal nitrogen concentration were recorded in *Chlorella protothecoides* and *Tetraselmis sp.* at 41.3 ppm and 61.6 ppm, respectively [50,51]. In fact, the accumulation of biomass and lipids in the mentioned two strategies is highly dependent on algal strains. Reports have highlighted the effect of nitrogen concentration on algal cell growth in different culture media [40,52,53].

Table 3. Summary of Nitrogen Stress Parameters.

No	Microalgal Strain	Temp. [°C]	Aeration Rate [L/min]	Culture Volume [L]	Optimal N Conc. [ppm]	Light Intensity [µmol/m²/s]	Ref
1	*Nannochloropsis oculata*	25	0.5	3	47.6	500	[45]
2	*Scenedesmus obtusus*	-	-	5	49.2	-	[46]
3	*Chlorella vulgaris*	25	0.2	1	43.4	60	[47]
4	*Chlorella vulgaris*	25	0.25	1	55.8	100	[48]
5	*Nannochloropsis oculata*	-	-	2	140.0	60	[54]
6	*Chlorella protothecoides*	30	2.5	5	41.3	-	[50]
7	*Acutodesmus dimorphus*	35	-	1	69.1	150	[55]
8	*Chlorella vulgaris*	25	-	6	41.3	70	[49]
9	*Tetraselmis sp.*	26	0.45	1	61.6	60	[51]

No	Before Nitrogen Stress				After Nitrogen Stress			
	Biomass	Lipid	Protein	Carbs.	Biomass	Lipid	Protein	Carbs.
1	2.3 g/L	10.0%	-	-	3.3 g/L	35.0%	-	-
2	2.8 g/L	22.6%	-	-	3.0 g/L	35.3%	-	-
3	3.5 g/L	25.3%	-	-	1.8 g/L	55.9%	-	-
4	1.87 g/L	15.5%	-	-	1.6 g/L	53.0%	-	-
5	0.66 g/L	10.0%	60.0%	28.0%	0.85 g/L	40.0%	40.0%	4.5%
6	82.6 g/L	20.0%	-	-	81.4 g/L	39.2%	-	-
7	0.35 g/L	24.3%	37.9%	21.2%	0.31 g/L	29.9%	26.9%	34.8%
8	0.51 g/L	9.6%	-	-	0.32 g/L	49.8%	-	-
9	0.9 g/L	21.0%	25.0%	25.0%	0.4 g/L	35.0%	40.0%	37.0%

2.6 Phosphorus Concentration

Phosphorus is another vital inorganic nutrient required for the growth of microalgae. Phosphorus is necessary for the formation of lipids, proteins and carbohydrates. Microalgae can assimilate inorganic phosphate compounds ($H_2PO_4^{2-}$ and HPO_4^{-}) to form organic compound through phosphorylation. Some microalgae strains can utilize phosphorus to form organic esters that are useful for cell growth. Moreover, phosphates can sediment at relatively high pH medium in the form of organic matter and phosphoric salts [56].

Excess phosphorus amount can be stored in the microalgal cells as polyphosphate granules that be used for the cell growth during the phosphate starvation conditions [57]. Accordingly, the phosphates reduction might distress the photosynthesis process and the total lipid production [58]. Various papers have reported the initial phosphorus concentration effects and phosphorus starvation on the biomass and lipid productivity [40,53,59].

2.7 Effect of Temperature

Temperature affects the metabolism and the ultimately biomass growth and production [25,60]. Indoor cultivation is preferred for controlling and adjusting the whole system to a desired temperature range. Table 4 is adopted from Ras et al. study which shows the temperature effect on microalgae [61]. The optimum values of temperature to attain optimal specific growth rate for different algal species is mentioned in Table 4..

Table 4. Optimum Temperatures and Growth Rates for Different Strain.

Microalgal Strain	$T_{Optimum}$ [°C]	T_{Max} [°C]	$\mu_{Optimum}$ [Day^{-1}]	Reference
D. Tertiolecta.	32.6	38.9	3.9	[62]
Chlorella pyrenoidosa	38.7	45.8	2.0	[63]
Nannochloropsis oceanica	26.7	33.3	1.8	[64]
P. Tricornutum	22.5	25.2	1.8	[65]
A. Formosa	20.1	29.8	1.6	[66]
Porphyridium cruentum	19.1	30.0	1.3	[67]
S. Costatum	24.5	33.0	1.0	[66]
Tychonema bourrelyi	21.8	30.0	1.0	[66]
Cryptomonas marssonii	15.9	30.3	0.8	[66]
Scenedesmus sp.	26.3	32.7	0.8	[68]
Dinobryon divergens	17.0	28.4	0.7	[66]
Ceratium furca	24.4	32.1	0.6	[69]
Ceratium fusus	26.5	30.7	0.5	[69]
Ceratium furcoides	22.3	30.0	0.3	[66]

2.8 Photosynthesis and Effect of Light Intensity

Photosynthesis is a distinctive process that converts energy of light and inorganic compounds to organic matter in photoautotrophs which depend on photosynthesis directly or indirectly as a source of energy for their growth and metabolism. Oxygenic photosynthesis can be expressed as light energy driven redox reaction in which water and carbon dioxide are converted into oxygen and carbohydrates. Two stages of conversion are typically occurred in oxygenic photosynthesis, namely light reactions and dark reactions. In light reactions, light energy is converted to high energy compound ATP and biochemical reductant $NADPH_2$. In dark reactions, ATP and $NADPH_2$ are utilized for biochemical reduction of CO_2 to carbohydrates. At low irradiance, the photosynthesis rate becomes linearly dependent on light intensity, while increasing light intensity reduces the photosynthesis process efficiency. Photosynthesis reaches maximum efficiency at optimal or saturation irradiance, but extended saturation irradiance decreases the rate of photosynthesis, and this phenomenon is known as photoinhibition [12]. Several parameters could affect the light efficiency such as cell pigmentation and culture density [70]. Indoor or artificial light source provides more irradiance density with an acceptable overall biomass productivity [31]. Many researchers have investigated different microalgal strains under different light intensities. Table 5 shows various lipid productivity profiles in *Chlorella vulgaris* strain growth under different light intensities. Gouveia and Oliveira [71] reported low lipid content and productivity but high biomass productivity at 150 µmol/m^2/s light intensity. However, at lower intensity of 75 µmol/m^2/s, Scragg et al. and Illman et al. [72,73]

reported high lipid content and productivity but low biomass productivity. Rodolfi et al. attained higher lipid and biomass productivity with light intensity of 100 µmol/m^2/s [74].

Table 5. Biomass and Lipid Productivities and Lipid Content of Different Microalgae Strains under Diverse Cultivation Light Intensities [31].

Microalgal Strain	Light Intensity [µmol/m^2/s]	Biomass Productivity [mg/L/d]	Lipid Content [%]	Lipid Productivity [mg/L/d]	Ref
Neochloris oleabundans	360.0	0.31–0.63	7.0–40.3	38.0–133.0	[75]
Chlorella sp.	300.0	0.37–0.53	32.0–34.0	121.3–178.8	[76]
Nannochloropsis oculata	300.0	0.37–0.48	22.7–29.7	84.0–142.0	[77]
Botryococcus braunii	150.0	0.027	20.8	5.5	[78]
Chlorella vulgaris	150.0	0.11	6.6	6.9	[78]
Chlorella vulgaris	150.0	0.18	5.1	7.4	[71]
Dunaliella tertiolecta	150.0	0.12	16.7	20	[71]
Dunaliella tertiolecta	150.0	0.1	60.6–67.8	60.6–69.8	[79]
Nannochloris sp.	150.0	0.04–0.35	29.9–40.3	15.6–109.3	[80]
Nannochloropsis sp.	150.0	0.09	28.7	25.8	[71]
Neochloris oleabundans	150.0	0.03–0.15	15.9–56.0	10.7–38.8	[81]
Neochloris oleabundans	150.0	0.09	29.0	26.1	[71]
Scenedesmus obliquus	150.0	0.09	17.7	15.9	[71]
Chaetoceros calcitrans	100.0	0.04	39.8	17.6	[74]
Chaetoceros muelleri	100.0	0.07	33.6	21.8	[74]
Chlorella sorokiniana	100.0	0.23	19.3	44.7	[74]
Chlorella sp.	100.0	0.23	18.7	42.1	[74]

Chlorella Vulgaris	100.0	0.20	18.4	36.9	[74]
Chlorococcum sp.	100.0	0.28	19.3	53.7	[74]
Ellipsoidion sp.	100.0	0.17	27.4	47.3	[74]
Isochrysis sp.	100.0	0.14	27.4	37.8	[74]
Monodus subterraneus	100.0	0.19	16.1	30.4	[74]
Nannochloropsis sp.	100.0	0.17	29.2	49.7	[74]
Nannochloropsis sp.	100.0	0.17	35.7	60.9	[74]
Pavlova lutheri	100.0	0.14	35.5	50.2	[74]
Pavlova salina	100.0	0.16	30.9	49.4	[74]
Skeletonema sp.	100.0	0.09	31.8	27.3	[74]
Tetraselmis sp.	100.0	0.30	14.7	43.4	[74]
Tetraselmis suecica	100.0	0.28	12.9	36.4	[74]
Thalassiosira pseudonana	100.0	0.08	20.6	17.4	[74]
Chlorella emersonii	76.0	0.04	25.0–34.0	10.3–12.2	[72]
Chlorella emersonii	76.0	0.03–0.05	29.0–63.0	8.1–49.9	[73]
Chlorella minutissima	76.0	0.02–0.03	31.0–57.0	9.0–10.2	[73]
Chlorella protothecoides	76.0	0.002–0.02	11.0–23.0	0.2–5.4	[73]
Chlorella sorokiniana	76.0	0.003–0.005	20.0–22.0	0.6–1.1	[73]
Chlorella vulgaris	76.0	0.03–0.04	18.0–40.0	5.4–14.9	[73]
Chlorella vulgaris	76.0	0.02–0.04	28.0–58.0	11.2–13.9	[72]
Scenedesmus obliquus	75.0	0.06	12.7	7.14	[82]

2.9 CO_2 Fixation and Uptake Efficiency

Carbon dioxide fixation occurred in the dark reaction stage of photosynthesis. In this stage, ATP and $NADPH_2$ produced during light reaction stage will be utilized in the following biochemical reaction:

$$CO_2 + 4H^+ + 4e^- \xrightarrow[3\ ATP]{\text{Enzymes}\\ 2\ NADPH_2} CH_2O + H_2O$$

The carbon fixation reaction mechanism was developed by Calvin and Benson using the ^{14}C radiolabeling method [12]. Caron dioxide is used as carbon source for the cultivation of phototrophic microalgae. CO_2 can be supplied to the culture either by a using pure source or flue gases, which partially can contribute to reduce CO_2 emissions and global warming. CO_2 sparging in the algal culture can decrease bubble size for better CO_2 uptake. Hence, larger bubbles formation reduces CO_2 utilization efficiency [25]. Several researches have been reported the fixation rate and CO_2 concentration in various microalgal species as shown in Table 6.

Table 6. Carbon Fixation Capacity under High CO_2 Concentration [83].

Microalgal Strain	CO_2 Concentration [%]	Fixation Rate [g/L/d]	Ref
Spirulina sp.	6.0	0.22	[84]
Chlorella sp. (mutant)	15.0	1.54	[85]
Haematococcus pluvialis	15.0	2.57	[86]
Aphanothece microscopica nägeli	15.0	5.60	[87]
Chlorella sp.	15.0	6.88	[76]
Scenedesmus obliquus	18.0	0.26	[84]
Scenedesmus sp.	20.0	0.42	[88]
Nostoc flagelliform	20.0	0.17	[89]
Chlorococcum littorale	40.0	1.00	[90]

2.10 Microalgal Major Components

Microalgae mainly consist of three major biochemical components namely carbohydrates, proteins, lipids and inorganic materials. The basic elements are carbon, nitrogen, oxygen and hydrogen that can be converted into biofuels which is an essential and remarkable characteristic of microalgae. Microalgae with high lipid content are required for the conventional technology of transforming microalgae to biofuel, while strains with low lipid contents have higher biomass productivity [91]. Microalgal biomass can be thermally converted to bio-gas products and can be used to produce ethanol as bio-product using fermentation process [92]. Microalgal components are dependent on cultivation environment, conditions and strain type.

2.10.1 Lipids

Lipids are substances that are soluble in non-polar solvents. It can be classified into neutral and polar lipids. Neutral lipids in microalgae are mainly composed of triglycerides, hydrocarbons, vitamins and pigments. Polar lipids found in the membranes of microalgae mainly composed of glycolipids and phospholipids. Fatty acids are the base molecules that form both neural and polar lipids. Fatty acids are composed of a hydrophilic carboxyl group attached to an unsaturated or saturated hydrophobic chain of hydrocarbon. Maximizing the lipid productivity and content can be achieved to improve biodiesel production and high value chemicals [12]. Researches have studied several microalgae strains with different conditions, lipid productivities and contents. Table 7 shows several microalgae strains with different lipid content.

2.10.2 Carbohydrates

Starch, glucose, sugars and polysaccharides are different chemical forms of carbohydrates that can be presented as microalgae biomass. Microalgal strains can produce extensive quantities of carbohydrates which can be used as a promising opportunity in commercialization [93]. Table 7 shows different microalgal strains with different carbohydrates content.

2.10.3 Proteins

Proteins are responsible to build the structure and to control metabolism of microalgae. For their metabolic activities, proteins regulate metabolic functions which enhance the microalgal growth. Proteins function as structure of microalgae can be noticed when chlorophyll molecules are synthesized under visible light [93]. Proteins depend on the composition and content of amino acids. Table 7 shows different microalgal strains with different proteins content. The recommended Kjeldahl conversion factor of 5.95 for elemental nitrogen was used to estimate the protein content [94].

Table 7. Main Biomass Compositions of Various Microalgal Species [91].

Microalgal Strain	Protein	Carbs.	Lipid	Ref
Nannochoropsis oculata	57	8	32	[95]
Dunaliella tertiolecta	64	21	15	[96]
Chlorella pyrenoidosa	71.3	22	0.1	[97]
Aphanizomenonflos	62	23	3	[98]
Chlamydomonas rheinhardii	48	17	21	[98]
Anabaena cylindrica	43–56	25–30	4–7	[98]
Dunaliella salina	57	32	6	[98]
Euglena gracilis	39–61	14–18	14–20	[98]
Spirogyra sp.	6–20	33–64	11–21	[98]
Porphyridiumcruentum	28–39	40–57	9–14	[98]
Scenedesmus obliquus	50–56	10–17	12–14	[98]
Spirulina maxima	60–71	13–16	6–7	[98]
Spirulina platensis	46–63	8–14	4–9	[98]
Chlorella protothecoides	53	11	15	[99]
Chlorella sp.	30	15–17	9–13	[100]
Chlorella vulgaris	42–58	12–17	14–22	[101]
Spirulina	55–70	17–23	4–13	[101]
Cladophora sp.	25	25	6	[102]
Lyngbya sp.	30	13	1	[102]
Desmodesmus sp.	38–44	13–20	10–14	[103]
Synechoccus sp.	63	15	11	[104]
Tetraselmismaculata	52	15	3	[104]
Dunaliella bioculata	49	4	8	[104]
Prymnesiumparvum	28–45	25–33	22–38	[104]
Scenedesmus quadricauda	47	—	2	[104]
Scenedesmus dimorphus	8–18	21–52	16–40	[104]
Chlorococcum littorale	38	23	16	[105]
Microcystisaeruginosa	31	12	13	[106]
Nannochloropsis salina	37	33	12	[107]
Nannochloropsis sp.	52	12	28	[108]
Scenedesmus sp.	60	10	20	[109]
S. Platensis	48	30	13	[110]

2.11 Biofuel Production

Microalgae have the potential to be an alternative, sustainable and clean source of energy due to their high growth rate and lipid content. Lipid extraction from algal biomass is quite difficult due to their thick cells wall that hinder the lipid from diffusing out of the cells. Mechanical pressure has no significant effect on lipid extraction from algal biomass. Organic solvents or supercritical fluids can be employed to extract lipid from microalgal cells. Extracted lipids can be further used for biodiesel production via transesterification reaction of lipids. In this reaction, triglyceride lipids react with alcohols in presence of a heterogenous or homogenous catalyst which then produce fatty acid methyl ester (FAME), glycerol, unreacted lipids and excess alcohol. Pretreatment, organic solvent lipid extraction, liquid-solid separation and purification are required for such process of lipid extraction by organic solvents [111].

2.11.1 Cell Disruption

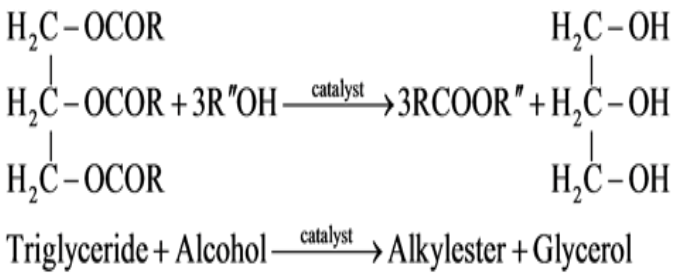
Overall mechanism of extraction process is controlled by diffusion as a rate controlling factor. Cell disruption can enhance and improve the diffusion efficiency of solvents [111]. Cell disruption avoid high temperature and pressure operating conditions [112]. Cell disruption is categorized into mechanical and non-mechanical methods. Mechanical methods include cavitation, sonication or autoclaving, while non-mechanical methods include osmotic shock, freezing or chemical methods [112].

2.11.2 Organic Solvent

Lipids are very highly soluble in organic solvents. Lipids extracted from microalgal cells contain neutral and polar lipids. Neutral lipids are hydrophobic molecules that interact with non-polar organic solvents such as chloroform, hexane, diethyl ether and benzene, while the polar lipids interact with polar organic solvents such as alcohols and dimethyl sulfoxide (DMSO) [113]. Organic solvent selection depends on several preferred characteristics such as selectivity of desired extracted components, volatility, immiscibility in water, level of toxicity and overall cost. For example, methanol, hexane and chloroform are highly toxic solvents that may cause serious health issues comparatively to ethanol which has a low level of toxicity. However, employing ethanol in lipid extraction in presence of water may decrease the efficiency of extraction due to azeotrope formation at specific concentrations [111]. Otherwise, accelerated solvent extraction (ASE) can utilize organic solvents at temperatures higher than boiling temperatures which boosts the lipid extraction [113]. Recently, ionic liquids are used as co-solvents with the organic solvents for lipid extraction from microalgal cells. Ionic liquids have some unique properties like low level of toxicity and vapor pressure. Further research is needed to investigate the optimal and efficient application of lipid extraction by organic solvents and ionic liquids as co-solvents [114]. Frequently, well-known techniques of lipid extraction from microalgal biomass are employed in laboratories such as Soxhlet (1879), Folch (1957), Bligh and Dyer (1959), ASE (1998) and Bigogno (2002) extraction techniques [12].

2.11.3 Transesterification

The biochemical conversion of algal oil into biofuels can be achieved by transesterification technique. Transesterification is a reaction between short chain alcohols and lipids that contain primarily triglycerides to produce FAME then biodiesel. The reaction takes place in the presence of heterogenous or homogenous catalyst. The most common alcohol used for lipid transesterification to produce biodiesel is methanol. The following equation represents the transesterification reaction:



Excess usage of alcohol might decrease the energetic efficiency and quality of the products. Therefore, alcohol amount must be controlled because excess alcohol could shift the reaction towards dehydrogenation reaction [115].

2.11.4 Interesterification

Interesterification is a reaction between triglyceride and another ester that contains fatty acid group to produce biodiesel and another triglyceride. The interesterification reaction was studied by using methyl acetate with marine *Chlorella salina* and *Nannochloropsis oculata* in presence of immobilized bio-catalyst where they achieved a biodiesel yield of 85.3 and 95.7%, respectively [116]. This reaction looks promising; however, extensive researches are needed to check its feasibility for scale-up employment.

CHAPTER 3

MATERIALS AND METHODS

3.1 Medium Preparation

At the first phase of this research, three different media were prepared, autoclaved and tested for three different strains. Each medium has its own recipe to provide certain nutrients to the microalgae for cell growth and cultivation. Firstly, stock solutions were prepared in 250 mL Erlenmeyer flasks. Then, certain amount of each stock solution was added and filled with deionized water up to 1 L in Erlenmeyer flask. After that, the prepared medium solutions were autoclaved at 121 °C for 3 hours then allowed to be cooled at room temperature before starting cultivation. Sterilization is needed at this stage to eliminate bacteria and most of the biological sources of contamination [117,118]. The following recipes were prepared to observe the isolated microalgae growth rate in a visual manner.

Table 8. Bold's Basel Medium Recipe.

Medium	BBM					
Flask Volume	1000.0 mL					
Component	**MW [g/mol]**	**Grams**	**Volume**	**Initial Conc.**	**Final Volume**	**Final Conc.**
$NaNO_3$	84.995	6.250	250.0	2.94E-04	10.0 mL	2.94E-03
$MgSO_4.7H_2O$	246.465	1.875	250.0	3.04E-05	10.0 mL	3.04E-04
NaCl	58.443	0.625	250.0	4.28E-05	10.0 mL	4.28E-04
K_2HPO_4	174.174	1.875	250.0	4.31E-05	10.0 mL	4.31E-04
KH_2PO_4	136.084	4.375	250.0	1.29E-04	10.0 mL	1.29E-03
$CaCl_2.2H_2O$	147.016	0.625	250.0	1.70E-05	10.0 mL	1.70E-04
H_3BO_3	61.832	2.850	250.0	1.84E-04	1.0 mL	1.84E-04
EDTA Solution	NA	NA	NA	NA	1.0 mL	NA
Ferric Solution	NA	NA	NA	NA	1.0 mL	NA
Trace Metals	NA	NA	NA	NA	1.0 mL	NA
Agar (Solid)	NA	NA	NA	NA	25.0 g/L	NA

Table 9. Cyanosite Medium Recipe.

Medium	SN					
Flask Volume	1000.0 mL					
Component	**MW [g/mol]**	**Grams**	**Volume**	**Initial Conc.**	**Final Volume**	**Final Conc.**
$NaNO_3$	84.995	76.50	1000.0	2.94E-04	30.6 mL	9.00E-03
K_2HPO_4	174.174	15.68	1000.0	4.31E-05	2.09 mL	9.00E-05
$EDTA.Na_2.2H_2O$	372.240	5.58	1000.0	1.50E-05	1.0 mL	1.50E-05
Na_2CO_3	105.988	10.70	1000.0	1.01E-04	1.0 mL	1.01E-04
Trace Metals	NA	NA	NA	NA	1.0 mL	NA
Agar	NA	NA	NA	NA	20.0 g/L	NA

Table 10. Artificial Seawater Medium Recipe.

Medium	ASW					
Flask Volume	1000.0 mL					
Component	**MW [g/mol]**	**Grams**	**Volume**	**Initial Conc.**	**Final Volume**	**Final Conc.**
$NaNO_3$	84.995	10.00	100.0	2.94E-04	40.0 mL	1.18E-02
$MgSO_4.7H_2O$	246.465	26.00	100.0	1.05E-03	10.0 mL	1.05E-02
NaCl	58.443	18.00	1000.0	NA	18.0 g/L	3.08E-01
KH_2PO_4	136.084	0.50	100.0	1.29E-04	2.86 mL	3.67E-04
$CaCl_2.2H_2O$	147.016	3.00	100.0	2.04E-04	10.0 mL	2.04E-03
NH_4Cl	53.492	2.70	100.0	5.05E-04	1.0 mL	5.05E-04
KCl	7.46E+01	6.00	100.0	8.05E-04	10.0 mL	8.05E-03
P-II Metal	NA	NA	NA	NA	10.0 mL	NA
Chelated Iron	NA	NA	NA	NA	1.0 mL	NA
Agar	NA	NA	NA	NA	20.0 g/L	NA

3.2 Microalgae Isolation

Three different samples from Al-Jubail coast were prepared and provided from the biology department at KFUPM. The expected strains in these samples were S1, S2 and S3 [119]. Agar plates were prepared by adding 20 to 25 grams of Agar.Agar nutrition powder for each liter of medium. Streaking technique was implemented under the biosafety cabinet for the isolation and purification of algal strains. This technique requires a sterile loop to select clear green growing algae cells from the samples. After that, streaking was employed by spreading the cells in a zigzag pattern on the agar plates. Considering two weeks of cultivation, single isolated portion was selected and streaked again on a new agar plate. Finally, in each streaking session, the colony was observed under the light microscope using glass slide and slide cover. The light microscope is used to check the axenic colony nature based on the color, shape and size [120].

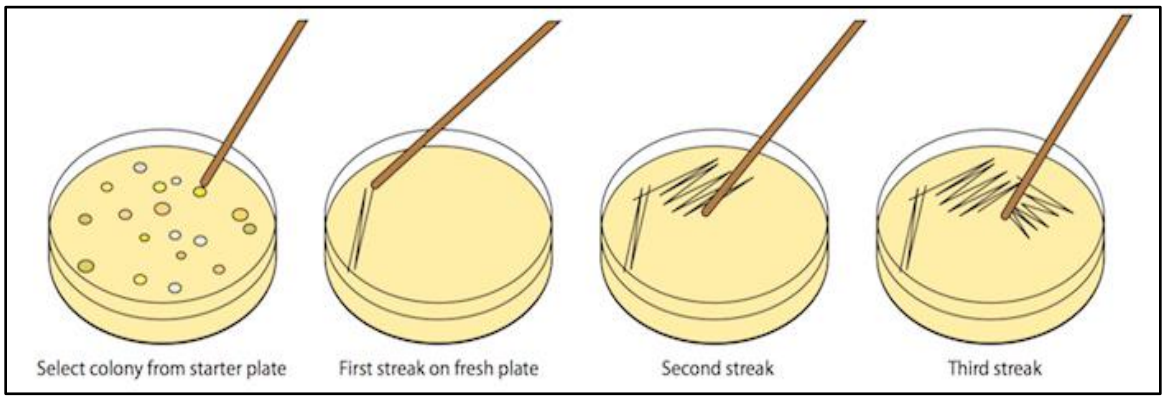


Figure 6. Streaking Technique Step by Step

3.3 Growth Analysis

3.3.1 Dry Weight

Measuring the dry weight of a sample is an accurate technique to determine the algal biomass. This technique is implemented by taking culture suspension aliquots then drying the samples to a constant weight and recording them as the dried cell weight per sample volume [121]. Nevertheless, Washing the marine algal samples with deionized water is mandatory for the determination of dry weight because the marine algal dry weight is affect by the extent of absorbed salts on the surface of the cells [122]. Firstly, a glass fiber filters were pre-weighted (Whatman GF/F, 47 mm, nominal pore size 0.7 μm) with aluminum weighing dishes. After that, a 20 mL of algal suspension aliquot was filtered by using a vacuum pressure between 33 to 55 mmHg. Then, the wet algal biomass was rinsed with deionized water. Next, the filtered algal biomass was dried at 105 °C to a constant weight and weigh with a microbalance [12]. Finally, the drying weight is calculated as:

$$\mathrm{DW}\ [\mathrm{mg/L}] = \frac{(\mathrm{m_{Total}} - \mathrm{m_{Filter}} - \mathrm{m_{Aluminum}})}{\mathrm{V_{Sample}}}$$

3.3.2 Optical Density

Accurate and rapid algal biomass concentration measurement is compulsory to analyze and determine the growth environment and stages of a culture in microalgae cultivation, biotechnology and algal physiology researches [123]. Optical density (OD) can be introduced and used as an advanced and alternative technique comparing to the time-consuming cell counting technique. OD, which is also acknowledged as absorbance (wavelength 600 to 750 nm), is a nondestructive, convenient, although indirect, and rapid technique to measure biomass concentration in algae cultures. The absorbed light by a cell suspension sample can be correlated directly to the biomass of cells [124]. Firstly, algal cultures were diluted to a concentration that fall within the linear range of the measurement (Value at 680 and 750 nm OD $\leq$ 1). After that, 2.0 mL sample cuvette was placed inside the spectrophotometer then data was recorded and saved in the system. Finally, correlation curve was constructed between OD values and dry weight biomass. To measure the algal biomass in batch cultures successfully through OD technique, it requires to generate diverse standard curves throughout the growth cycle.

3.4 Nitrate Measurement

Nitrate was measured by analyzing the total nitrate using Hach nitrate kit and standard chromotropic acid method by DR3900 spectrophotometer. Firstly, a culture sample of 2.0 mL was centrifuged at 5000 rpm for 5 minutes. Then, the centrifuged sample supernatant was transferred to a new 2.0 mL vial. After that, a volume of 1.0 mL of the supernatant was diluted to a certain factor with deionized water. DR3900 spectrophotometer was operated and (344 N. Nitrate HR, TNT) stored program was selected at wavelength of 410 nm. Then, a diluted sample of 1.0 mL was added to the (NitraVer X Reagent A) testing tube and inverted ten times to guarantee proper mixing. After that, the testing tube was wiped and put to zero by pressing the zero button. Afterward, powder pillow of (NitraVer X Reagent B) was added and the tube was mixed and inverted ten times then kept for 5 minutes to completely react with the reagents. Finally, the tube was wiped and placed in the cell holder of the DR3900 spectrophotometer for the nitrate measurement and the reading was displayed in mg/L [125]. The total nitrogen removal efficiency can be estimated by the following equation:

$$TNR = \frac{TN_i - TN_t}{TN_i}$$

3.5 Phosphorus Measurement

Phosphorus was measured by analyzing the total phosphorus using Hach nitrate kit, digital reactor DRB200 for coking and standard molybdovanadate method with acid persulfate digestion by DR3900 spectrophotometer. Firstly, a culture sample of 5.0 mL was centrifuged at 5000 rpm for 5 minutes. Then, the centrifuged sample supernatant was transferred to the kit vials. DR3900 spectrophotometer was operated and (542 P. Total HR, TNT) stored program was selected at wavelength of 410 nm. Then, sample of 5.0 mL was added to the (Total High Range Phosphorus Test 'N Tube Reagent Set) testing tube and inverted ten times to guarantee proper mixing. Afterward, powder pillow of (potassium persulfate) was added and the tube was mixed and inverted ten times then kept inside the digital reactor for 30 minutes at 150 °C. The sample then was cooled to room temperature. After that, 2.0 mL of 1.54 N sodium hydroxide standard solution was added to the vial and mixed for 30 seconds and then 0.5 mL of molybdovanadate reagent was added to the vial with proper inverting and mixing. Finally, the sample kept for 7 minutes to completely react with the reagents. Finally, the tube was wiped and placed in the cell holder of the DR3900 spectrophotometer for the phosphorus measurement and the reading was displayed in mg/L [126]. The total phosphorus removal efficiency can be estimated by the following equation:

$$TPR = \frac{TP_i - TP_t}{TP_i}$$

3.6 Total Lipids Measurement

The analysis of total lipids is performed by three consecutive stages, biomass harvesting, freeze-drying, lipid extraction and quantification.

3.6.1 Biomass Harvesting

At the end of the cultivation process, cultures were collected and centrifuged at 5000 rpm for 5 minutes using HITACHI-CR22-GIII device. After that, the wet biomass was rinsed with deionized water to reduce salts content then centrifuged again. This process was repeated three times to ensure less salts content presented in the total lipid analysis.

3.6.2 Lyophilization

Freeze drying or lyophilization is the process of removing the ice or frozen solvents from the wet algal biomass through sublimation and desorption phenomena. The harvested and centrifuged biomass was frozen up to -35 °C by using a deep freezer. After that, the frozen biomass was loaded in a VirTis flask and freeze-dried under vacuum (≤ 4.5 Torr) at -40 °C using VirTis freeze dryer. Finally, the dried biomass was removed as dried product.

3.6.3 Total Lipid Extraction

At this stage, Bligh–Dyer technique is used for the total lipid extraction from dried microalgal biomass. Initially, a 5.0 mL glass vial with PTFE cap was pre-weighted using a microbalance. Then, 50 to 70 milligrams of dried microalgal biomass were weighted and loaded into the glass vial with 0.4 mL buffer solution. After that, 1.5 mL of mixed solution of (chloroform-methanol, 2:1 v/v) was added into the glass vial and sonicated for 30 minutes at 60 °C. A volume of 1.5 mL of chloroform was added and thoroughly mixed for 5 minutes again. Afterward, 0.5 mL of deionized water were added and vortexed for another 5 minutes. Next, the glass vial was centrifuged at 4500 rpm for 10 minutes to produce a lower organic layer and an upper aqueous layer with white precipitate that regularly exists at the interface. Finally, the organic layer was transferred to a new glass vial and solvents were evaporated by using a nitrogen evaporator [127]. The extracted lipid content was dried, and the glass vial was weighted to calculate the lipid content as follows:

$$\text{Lipid Content } [\%] = \frac{(m_{\text{Extracted Lipid}} - m_{\text{Glass Vial}})}{m_{\text{Dry Biomass}}}$$

3.7 FAME Analysis

3.7.1 First Method

For lipid analysis from harvested and dried biomass, around 10 mg of dry biomass were extracted and sonicated with 2.0 mL of 2:1 chloroform-methanol mixture in PTFE screw-capped Pyrex vials for 30 minutes at 45 °C. After that, the extracted lipids were converted to fatty acid methyl esters (FAME) by transesterification reaction. 1.0 mL of methanol was added to the extracted lipids with 0.3 mL of sulfuric acid as a catalyst, after which the transesterification reaction occurred for 10 min at 60 °C. After the reaction, the samples cooled to room temperature then 1.0 mL of demineralized water was added to wash out the residual sulfuric acid and methanol. For phase separation, samples were centrifuged at 4500 rpm for 3 minutes. The lower organic layer in chloroform phase was separated and filtered through a 0.2 µm PVDF syringes filter. The FAME in the organic phase were analyzed by gas chromatography mass spectrometry (GC-MS, GC-6890N, MSD-5975B, Agilent Technologies, USA). Each FAME components were identified and quantified by comparing the peak areas and retention times with the food industry FAME mix by RESTEK.

3.7.2 Second Method

For lipid analysis from harvested and dried biomass, around 10 mg of dry biomass were extracted and sonicated with 2.0 mL of 9:1 methanol-dimethyl sulfoxide mixture in PTFE screw-capped Pyrex vials for 30 minutes at 45 °C. After that, the extracted lipids were converted to fatty acid methyl esters (FAME) by transesterification reaction. 1.0 mL of 1:1 hexane-diethyl ether mixture was added to the extracted lipids with 0.3 mL of sulfuric acid as a catalyst, after which the transesterification reaction occurred for 10 min at 60 °C. After the reaction, the samples cooled to room temperature then 1.0 mL of demineralized water was added to wash out the residual sulfuric acid. For phase separation, samples were centrifuged at 4500 rpm for 3 minutes. The upper organic layer was collected in a new vial then lipids were re-extracted by adding 1.0 hexane-diethyl ether mixture. After collecting the extracted organic layers, the FAME in the organic phase were analyzed by gas chromatography mass spectrometry (GC-MS, GC-6890N, MSD-5975B, Agilent Technologies, USA). Each FAME components were identified and quantified by comparing the peak areas and retention times with the food industry FAME mix by RESTEK.

FAME concentrations were attained as a mass fraction in percent of the total sample weight using the following equation [128]:

$$\text{FAME}\ [\%] = \frac{\text{Area}_{\text{FAME}}}{\text{Area}_{\text{C17:0}}} \times \frac{C_{C17:0} \times V_{C17:0}}{\text{m}_{\text{Dry Biomass}}} \times 100$$

where: $\text{Area}_{\text{FAME}}$: is the total peak area, Area_{FM}: is the peak area corresponding to the pure FAME mix solution, C_{FM}: is the concentration, of the pure FAME mix solution (mg/ml), V_{FM}: is the volume of the pure FAME mix solution (ml) and $\text{m}_{\text{Dry Biomass}}$: is the mass of the dried microalgal sample (mg).

3.8 Total Fixed Carbon Determination

Total fixed carbon, hydrogen and nitrogen were determined by elemental analysis (PerkinElmer 2400 Series II CHNS/O Elemental Analyzer, Perkin Elmer Corporation). Harvested and dried biomass samples were weighted up to 0.8 to 2.0 mg in pre-weighted and precleaned tin capsules (5×8 mm, Perkin Elmer). The capsules were then combusted at 1000 °C using pure helium as the carrier gas and pure oxygen as the combustion gas. Carbon and hydrogen were determined by infrared absorption while nitrogen was determined as N_2 using a thermal conductivity detection system [94]. The instrument was calibrated with acetanilide standards with delta calibrated criteria of ±0.15 for carbon, ±3.75 for hydrogen and ±0.16 for nitrogen. The rate of CO2 capture can be estimated by the following equation [129]:

$$R_{CO_2} = P \cdot C_{CO_2} \cdot \frac{M_{CO_2}}{M_C}$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Medium and Strain Selection

At the start of this research, an experiment of selecting medium and microalgae strain has been investigated and tested under atmosphere aeration and fixed temperature of 25 °C for 21 days of cultivation. This experiment included three different mediums which are: BBM, SN and ASW. Each medium was tested for three different local isolated strains which are S1, S2 and S3. Each Erlenmeyer flask has a volume 125 mL with an actual working volume of 75 mL. Each flask was shaking roughly by hand two times a day. Light intensity of 40 µmol/m^2/s were provided to the flask with a continuous shaking of 110 rpm. The following figures shows the visual observation of the microalgae growing progression. S3 strain were identified as *Parachlorella kessleri* strain HY-6 with 18S ribosomal RNA gene by the Royal Life Sciences Pvt. Ltd. reference number 160609FN-119 (accession number JQ797561.1).

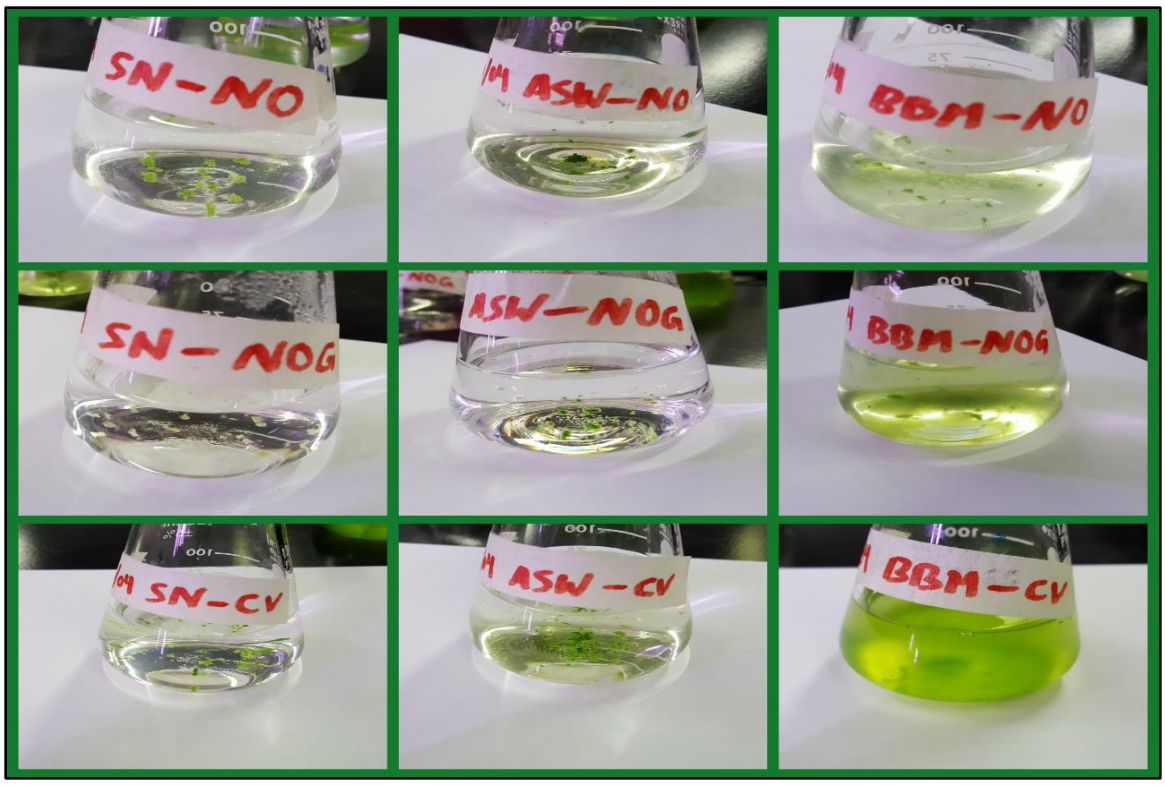


Figure 7. Growth Visual Observation at Day 7.

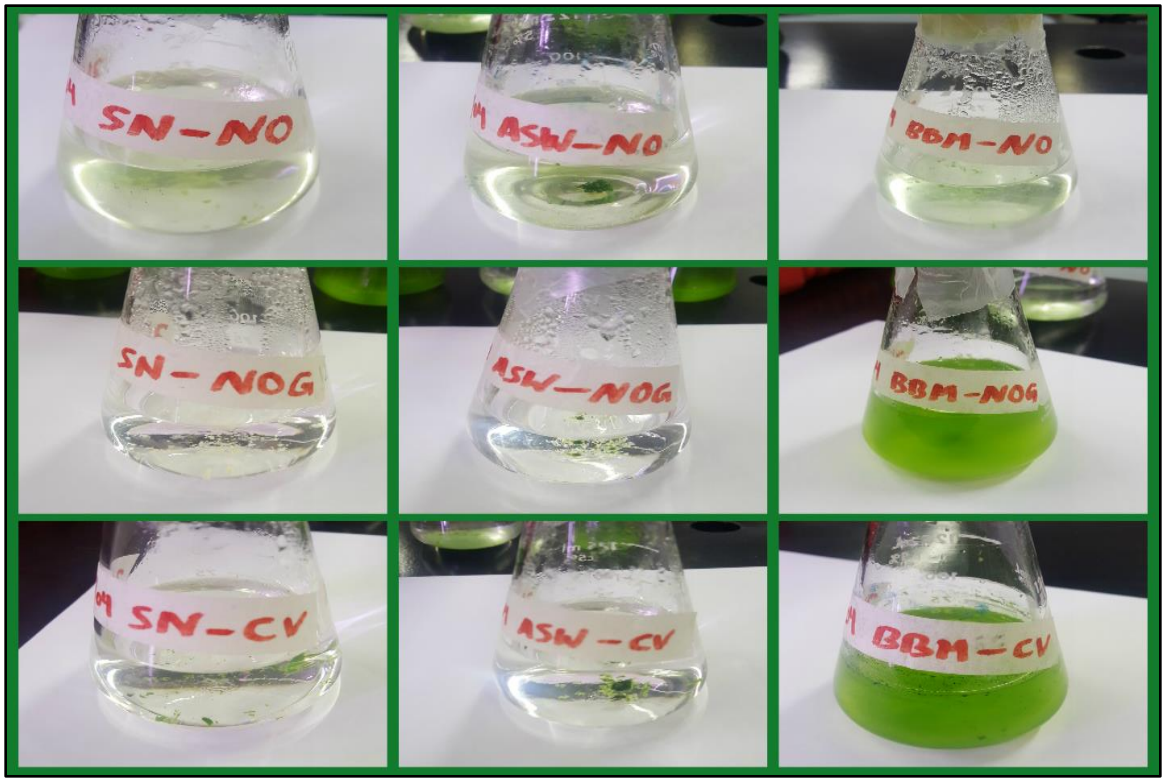


Figure 8. Growth Visual Observation at Day 14.

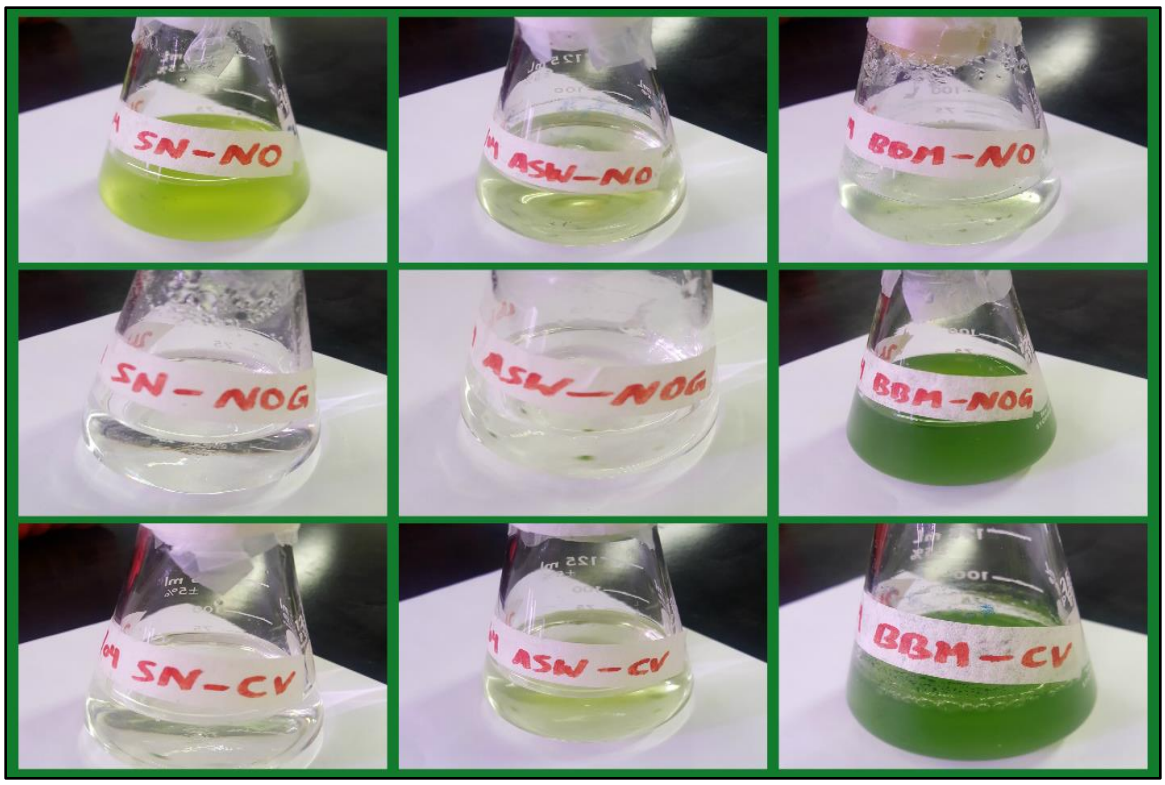


Figure 9. Growth Visual Observation at Day 21.

According to the visual growth observation of microalgae cells after 21 days of cultivation as summarized in Table 11, BBM medium showed high cell growth compared to SN or ASW mediums. S1 and S3 showed better growth in BBM medium.

Table 11. Results of Growth Observation for Different Microalgae & Mediums.

Microalgae Strain	Medium		
	BBM	SN	ASW
S1	+	++	+
S2	+++	-	-
S3	+++	-	+

In this research, *Parachlorella kessleri (*S3) local strain was selected to be cultivated and tested in BBM medium under varying nutrient concentrations, temperatures, light intensities and CO_2 fixation rates.

4.2 Standard Calibration Growth Curve

Standard calibration growth curve was generated for both strain *Chlorella kessleri* and *Parachlorella kessleri* to estimate the biomass concentration of the algal cells at any measured OD by the spectrophotometer. This curve is essential to estimate the biomass concentration then further the biomass productivity for the daily basis analysis. This curve was generated by diluting known amount of algal liquid culture up to hundred times then measure the OD and dry weight per volume for each diluted run. The following Figure 10 and 11 shows the result of the calibrated growth curve for cells concentration estimation which can be represented by a linear relationship between OD and DW.

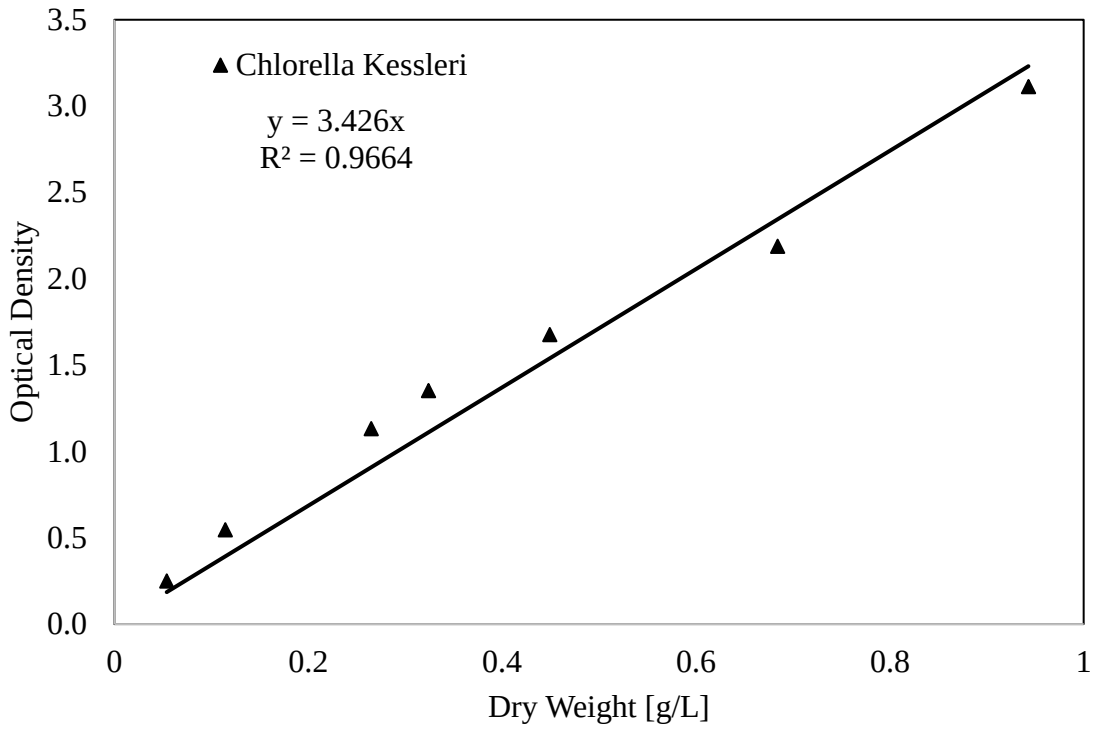


Figure 10. Standard Curve of Optical Density at 680 nm Versus Dry Weight of Diluted Chlorella Kessleri Culture.

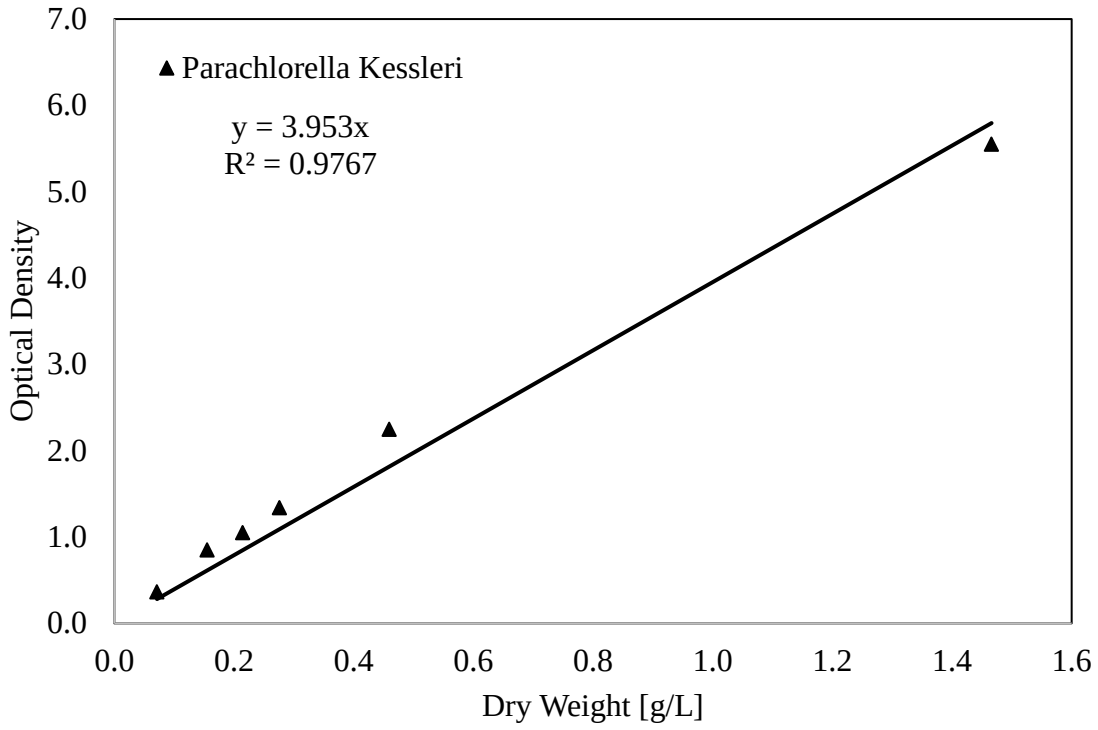


Figure 11. Standard Curve of Optical Density at 680 nm Versus Dry Weight of Diluted Parachlorella Kessleri Culture.

The relationship between OD and DW for each strain is summarized as follows:

For *Chlorella kessleri*: $OD_{680} = 3.426\ DW\ (R^2 = 0.9664)$

For *Parachlorella kessleri*: $OD_{680} = 3.953\ DW\ (R^2 = 0.9767)$

4.3 Optimum Nitrogen

4.3.1 Growth Kinetics

In the second stage of this research, an experiment of determining the optimum concentration of nitrate in the BBM medium has been performed and tested under atmosphere aeration,1.5% CO_2 Concentration, pH of 7.35 and fixed temperature of 25 °C for 16 days cultivation period. This experiment included five different initial nitrogen concentrations which are: 15.8, 31.6, 47.4 and 63.2 ppm. For data reliability and validity, each concentration was tested twice for the optimum growth of the local strain Parachlorella vulgaris. Each Erlenmeyer flask has a volume 500 mL with an actual working volume of 350 mL. Each flask was shaking by hands two times daily. Light intensities of 30 and 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ were provided to the flasks with a continuous shaking of 150 rpm. Table 12 below summarizes the characteristics of the same culture under different initial nitrogen concentrations and light intensities. Specific growth rate represents the increase of certain biomass from every gram of existing biomass per unit time which known as the average growth rate of all cells in a culture. Doubling time or generation time is defined as the required time to achieve a double amount of viable cells [12]. The growth curves of cell growth attained with different nitrogen concentrations are shown in Figure 12 and 13. As shown the cell growth of *Parachlorella kessleri* enhanced notably when the nitrogen concentration increased from 47.4 to 63.2 ppm. As summarized in Table 12, the specific growth rate, doubling time, biomass productivity and growth yield reached high and comparable values at 47.4 and 63.2 ppm, which were 0.3 day^{-1}, 2.3 day and 167 mg/L/day, respectively.

Table 12. Growth Characteristics of Parachlorella Kessleri at Different Initial Nitrogen Concentrations and Light Intensities.

TN	TP	Illuminance	μ_g	τ_D	Productivity	$Y_{X/S}$
[ppm]	[ppm]	[$\mu mol \cdot s^{-1} \cdot m^{-2}$]	[Day^{-1}]	[Day]	[mg/L/Day]	[mg /mg]
15.8	53.3	60	0.331	2.10	83.5	84.4
31.6	53.3	60	0.338	2.05	130.4	66.7
47.4	53.3	60	0.341	2.03	158.4	53.7
63.2	53.3	60	0.341	2.03	167.0	42.2
15.8	53.3	30	0.275	2.52	78.4	79.2
31.6	53.3	30	0.337	2.06	119.3	61.0
47.4	53.3	30	0.360	1.93	144.9	49.2
63.2	53.3	30	0.294	2.36	145.4	37.0

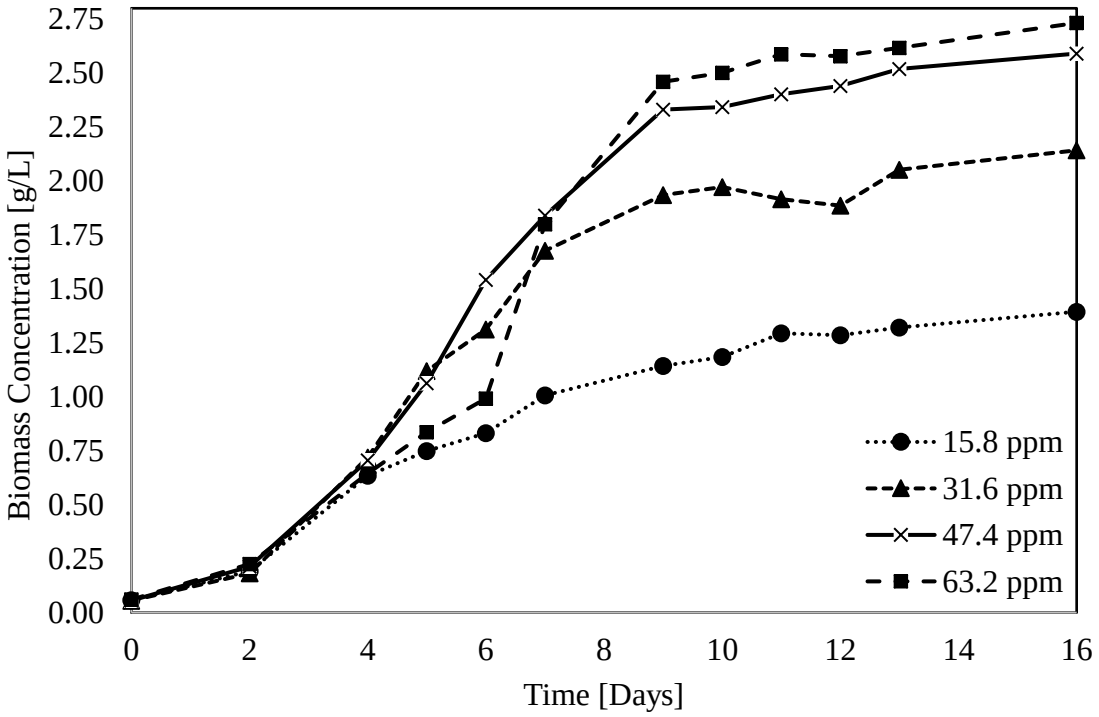


Figure 12. Growth of Parachlorella Kessleri at 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$ with Different Initial Nitrogen Concentrations.

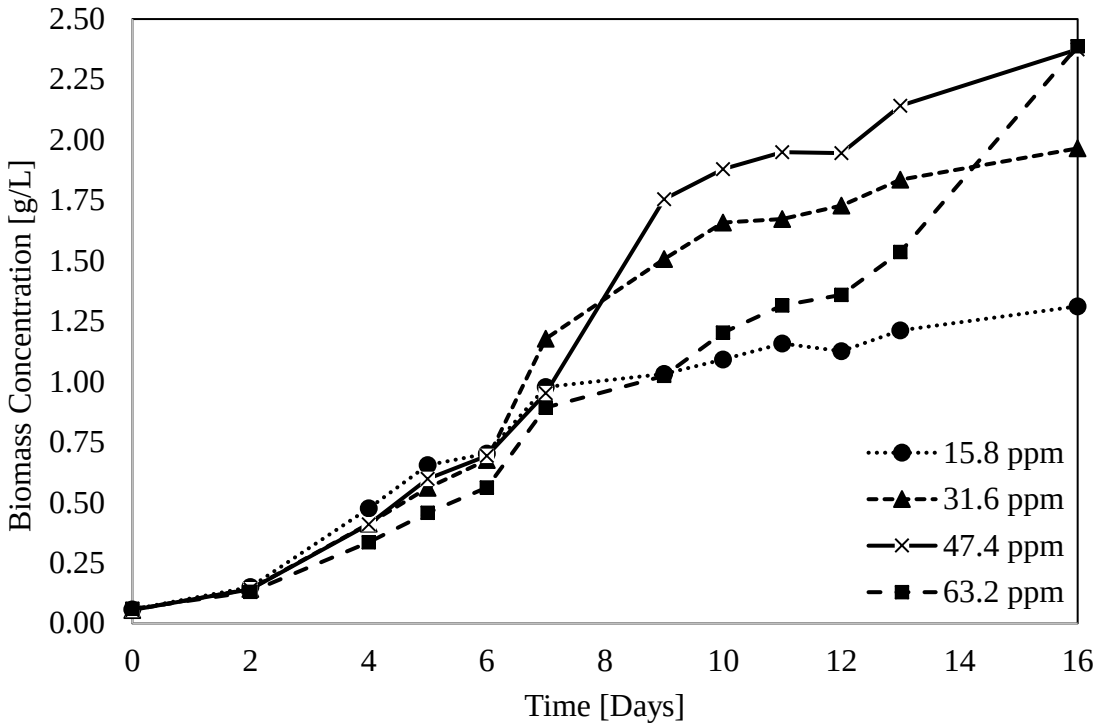


Figure 13. Growth of Parachlorella Kessleri at 30 µmol·s^{-1}·m^{-2} with Different Initial Nitrogen Concentrations.

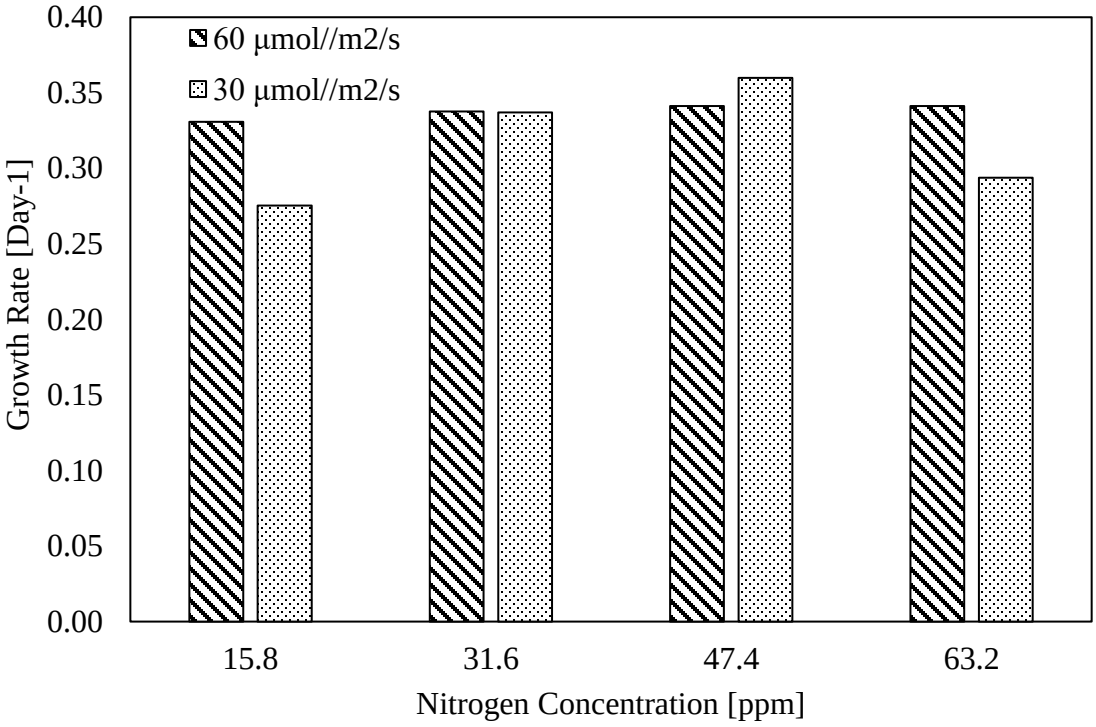


Figure 14. Growth Rate of Parachlorella Kessleri with Different Initial Nitrogen Concentrations and Light Intensities.

4.3.2 Nutrient Uptake and Removal

Nitrogen removal and uptake were measured as summarized in Table 13 to 16 and shown in Figure 15 and 16 for of 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (High Light) and 30 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (Low Light), respectively. Different initial concentrations where the nitrogen in media with 15.8, 31.6, 47.4, and 63.2 ppm was depleted after 2, 4, 4 and 5 days, respectively with a removal efficiency of TN >99%.

Table 13. TN Uptake by Parachlorella Kessleri with Different Nitrogen Concentrations at 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$.

Day	TN (ppm)			
	15.8 ppm	**31.6 ppm**	**47.4 ppm**	**63.2 ppm**
0	15.8	31.3	47.2	63.3
2	1.1	17.7	31.4	47.9
4	0.5	0.5	1.0	19.2
5	0.3	0.4	0.6	0.6
6	0.2	0.2	0.5	0.2
10	0.0	0.0	0.1	0.0
12	0.0	0.0	0.0	0.0
14	0.0	0.0	0.0	0.0
16	0.0	0.0	0.0	0.0

Table 14. TN Removal by Parachlorella Kessleri with Different Nitrogen Concentrations at 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$.

Day	Removal TN (%)			
	15.8 ppm	**31.6 ppm**	**47.4 ppm**	**63.2 ppm**
0	0.0	0.0	0.0	0.0
2	93.3	43.3	33.4	24.4
4	97.1	98.4	97.9	69.7
6	98.1	98.8	98.8	99.1
8	98.9	99.4	99.0	99.8
10	100.0	100.0	99.7	100.0
12	100.0	100.0	100.0	100.0
14	100.0	100.0	100.0	100.0
16	100.0	100.0	100.0	100.0

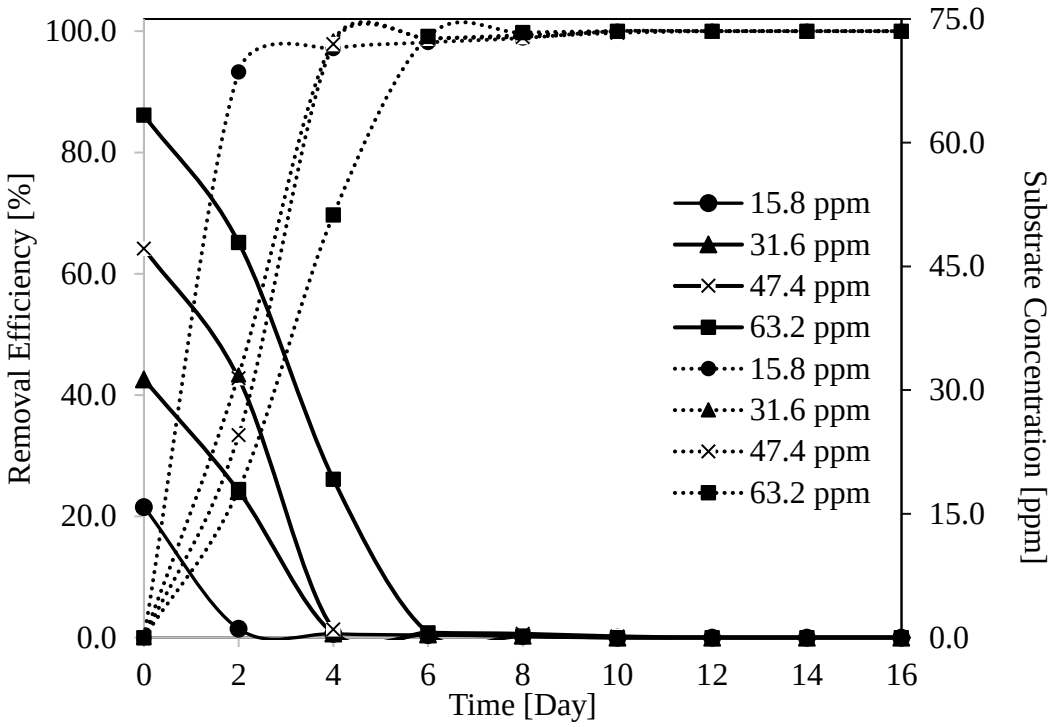


Figure 15. TN Removal and Uptake by Parachlorella Kessleri with Different Nitrogen Concentrations at 60 µmol·s^{-1}·m^{-2}.

Table 15. TN Uptake by Parachlorella Kessleri with Different Nitrogen Concentrations at 30 µmol·s^{-1}·m^{-2}.

Day	TN (ppm)			
	15.8 ppm	**31.6 ppm**	**47.4 ppm**	**63.2 ppm**
0	15.8	31.3	47.2	63.3
4	0.6	8.6	20.3	44.0
5	0.4	0.9	10.7	38.7
6	0.2	0.5	3.0	30.7
7	0.1	0.2	1.7	23.8
10	0.0	0.0	0.5	16.8
12	0.0	0.0	0.2	10.1
13	0.0	0.0	0.0	6.2
16	0.0	0.0	0.0	0.5

Table 16. TN Removal by Parachlorella Kessleri with Different Nitrogen Concentrations at 30 µmol·s^{-1}·m^{-2}.

Day	Removal TN (%)			
	15.8 ppm	31.6 ppm	47.4 ppm	63.2 ppm
0	0.0	0.0	0.0	0.0
2	96.4	72.5	57.1	30.5
4	97.3	97.0	77.2	38.9
6	98.4	98.3	93.5	51.5
8	99.1	99.4	96.5	62.5
10	100.0	100.0	98.9	73.5
12	100.0	100.0	99.5	84.0
14	100.0	100.0	100.0	90.2
16	100.0	100.0	100.0	99.2

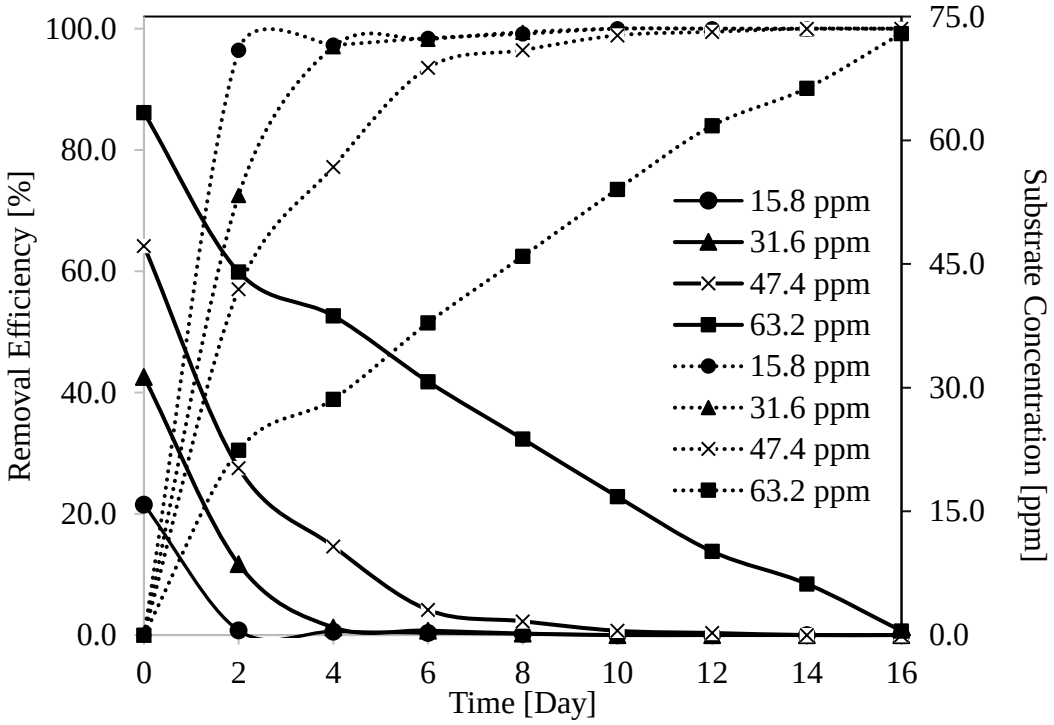


Figure 16. TN Removal and Uptake by Parachlorella Kessleri with Different Nitrogen Concentrations at 30 µmol·s^{-1}·m^{-2}.

4.3.3 Total Lipid Analysis

Table 17 shows the total lipid contents in dry cells, biomass and lipid productivity attained at different nitrogen concentrations. The lipid content decreased obviously when the nitrogen concentration increased in the range of 15.8 to 63.2 ppm from 46.7 to 26.5% for the high light at 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$, respectively. On other hand, lipid content reached 52.8% at 30 $\mu mol \cdot s^{-1} \cdot m^{-2}$. However, the maximum lipid productivity attained when the nitrogen concentration was 47.4 ppm with 56.2 mg/L/day at 60 $\mu mol/m^2/s$. According to these findings, we found that the optimal compromise between maximizing the cell biomass and lipid productivity occurs at 47.4 ppm of nitrogen concentration among the experimented conditions as can be seen in Figure 17.

Table 17. Lipid Content, Algal Biomass and Lipid Productivity of Parachlorella Kessleri with Different Initial Nitrogen Concentrations.

TN	Illuminance	Algal biomass	Lipid Content	Lipid Productivity
[ppm]	[$\mu mol \cdot s^{-1} \cdot m^{-2}$]	[g/L]	[%]	[mg/L/Day]
15.8	60	1.34	46.7	39.0
31.6	60	2.09	36.3	47.3
47.4	60	2.53	35.5	56.2
63.2	60	2.67	26.5	44.2
15.8	30	1.25	52.8	41.4
31.6	30	1.91	28.8	34.3
47.4	30	2.32	23.3	33.8
63.2	30	2.33	11.4	16.6

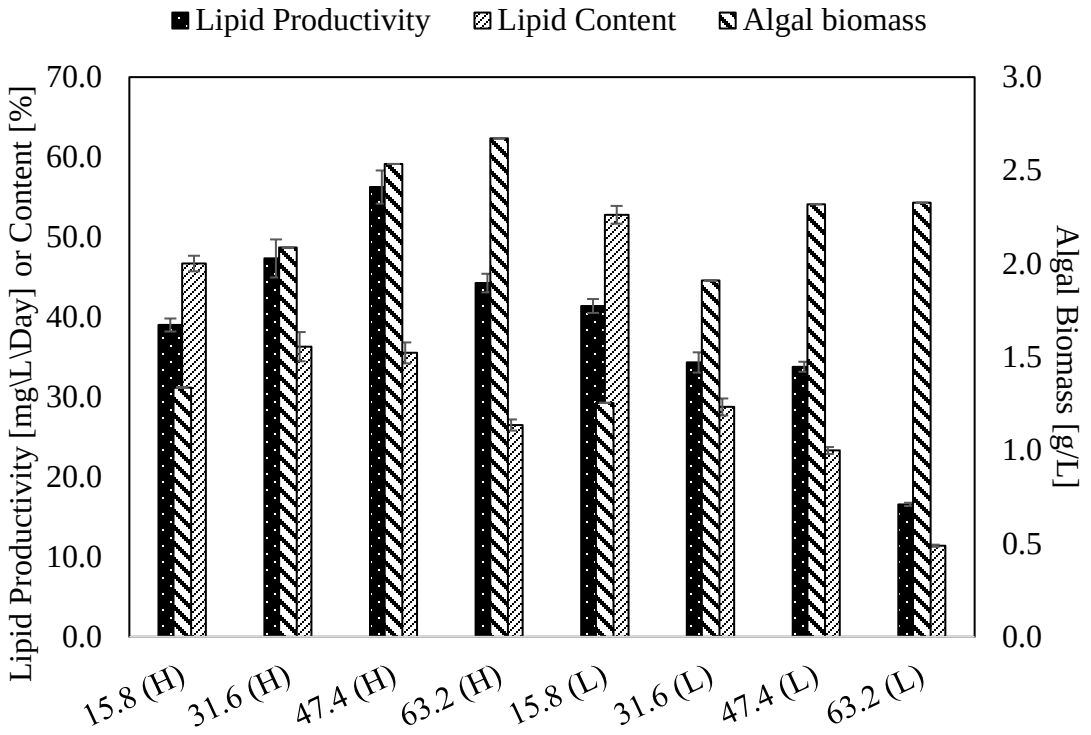


Figure 17. Lipid Content, Algal Biomass and Lipid Productivity of Parachlorella Kessleri with Different Initial Nitrogen Concentrations.

4.4 Nitrogen Starvation

4.4.1 Growth Kinetics

Nitrogen starvation is on the most effective strategies to stimulate high lipid content in microalgal culture [73]. High lipid productivity under nitrogen starvation condition can be obtained for algal based biofuel projections. After 10 cultivation days (stage I), the *Chlorella kessleri* cells were resuspended under light intensity of 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (High Light) and 30 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (Low Light) for 9 days with nitrogen-deficient system (stage II). Earlier studies mentioned that many algae strains adjust their lipid biosynthetic pathways for the accumulation and formation of neutral lipids, mainly in form of TAGs along monoacylglycerols, diacylglycerols and sterol esters under nitrogen starvation conditions [130]. Algal cells utilize chlorophylls as a nitrogen source under nitrogen starvation condition and probably transform proteins to TAGs or carbohydrates [75]. Under environmental stress like the nitrogen starvation, the fixed CO_2 in photosynthesis is diverted from protein synthesis path to that of carbohydrate or lipid synthesis [48]. Previous study stated that carbohydrate would be initially synthesized to reserve energy and then, as a long-standing storage mechanism, lipid would be produced in cases of expanded environmental stress [131]. As summarized in Table 18, the specific growth rate, doubling time, biomass productivity and growth yield reached high and comparable values at second cumulative second and first stage, which were 0.561 day^{-1}, 1.24 day and 224.9 mg/L/day, respectively.

Table 18. Growth Characteristics of Parachlorella Kessleri Before and After Nitrogen Starvation at Different Light Intensities.

Stage	Illuminance	μ_g	τ_D	Productivity	$Y_{X/S}$
	[µmol·s^{-1}·m^{-2}]	[Day^{-1}]	[Day]	[mg/L/Day]	[mg /mg]
I	60	0.513	1.35	179.1	38.0
I+II	60	0.561	1.24	224.9	-
I	30	0.388	1.79	148.6	32.8
I+II	30	0.461	1.50	211.2	-

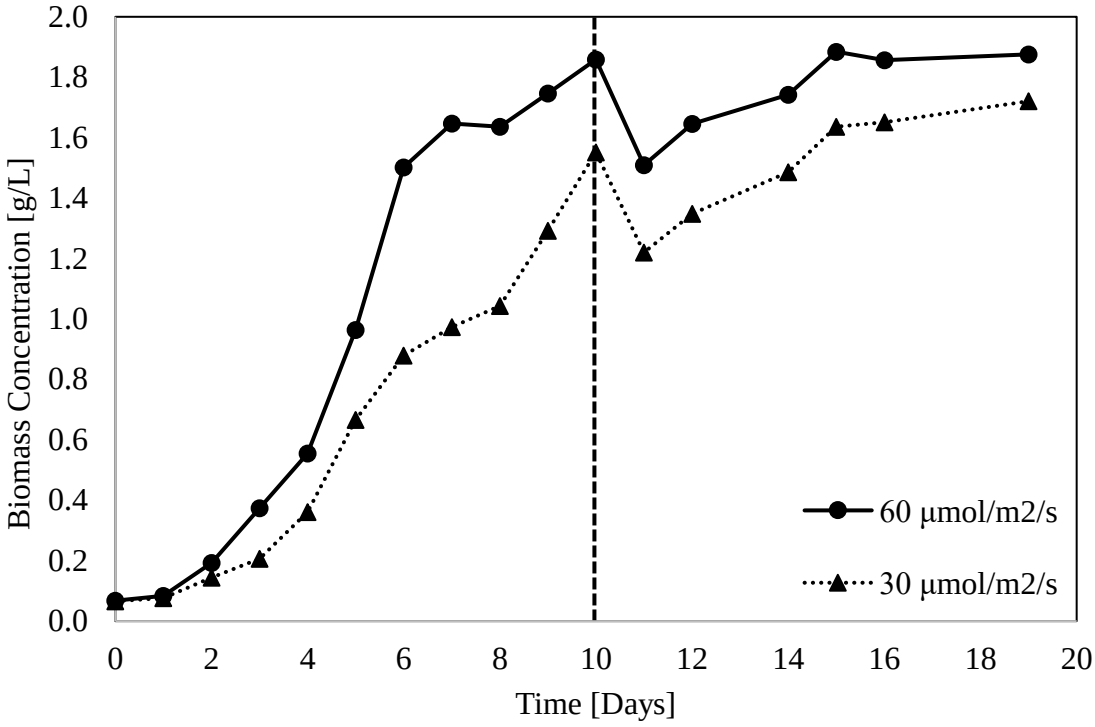


Figure 18. Growth of Parachlorella Kessleri with Nitrogen Starvation at Different Light Intensities.

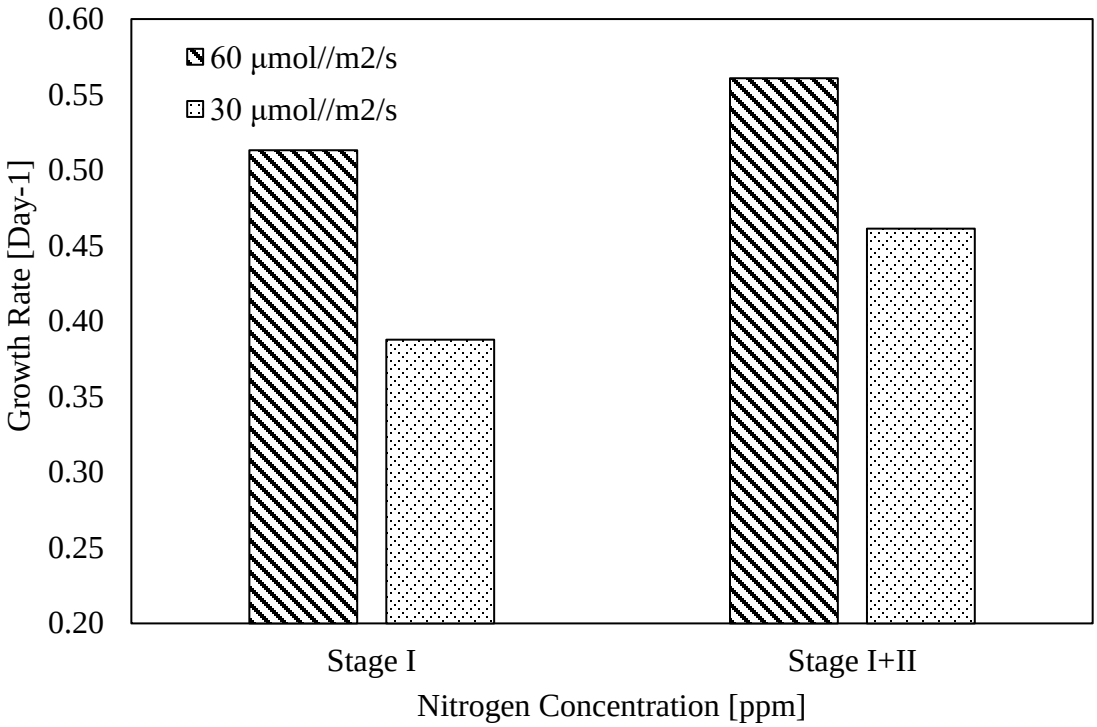


Figure 19. Growth Rate of Parachlorella Kessleri with Nitrogen Starvation at Different Light Intensities.

4.4.2 Nutrient Uptake and Removal

Table 19 and 20 shows removal efficiencies of total nitrogen and phosphorus where the nitrogen and phosphorus were depleted after 6 and 8 days, respectively with a TN removal efficiency of 97% and TP removal efficiency of 14%. Low TP removal efficiency is due to high NP ratio (1:1) that has been fixed at this experiment. To develop more feasible and economical production of microalgae-based biofuels, it is recommended to obtain the highest lipid content in consort with the highest lipid productivity simultaneously. Lower biomass productivity is generally linked with low overall lipid productivity. Thus, to achieve higher lipid productivity, further investigation is required for the period of nitrogen starvation and different NP ratios as will be discussed in the next section.

Table 19. TN and TP Uptake by Parachlorella Kessleri Before and After Nitrogen Starvation at Different Light Intensities.

Stage	Day	TN (ppm)		TP (ppm)	
		60 µmol·s^{-1}·m^{-2}	30 µmol·s^{-1}·m^{-2}	60 µmol·s^{-1}·m^{-2}	30 µmol·s^{-1}·m^{-2}
I	0	48.2	46.4	40.5	40.5
	1	39.8	42.0	-	-
	2	30.2	35.2	-	-
	3	14.8	26.8	-	-
	4	10.7	24.3	-	-
	5	4.2	21.4	-	-
	6	1.4	6.0	37.4	38.3
	7	1.1	1.8	-	-
	8	1.0	1.2	37.0	38.8
I+II	11	0.9	1.0	40.5	40.5
	12	0.9	0.9	38.2	38.2
	14	0.9	0.9	37.3	37.4
	15	1.8	2.0	37.2	37.1
	19	1.2	1.5	34.9	33.5

Table 20. TN and TP Removal by Parachlorella Kessleri Before and After Nitrogen Starvation at Different Light Intensities.

Stage	Day	Removal TN (%)		Removal TP (%)	
		60 µmol·s^{-1}·m^{-2}	30 µmol·s^{-1}·m^{-2}	60 µmol·s^{-1}·m^{-2}	30 µmol·s^{-1}·m^{-2}
I	0	0	0	0.0	0.0
	1	17.3	9.3	-	-
	2	37.4	24.0	-	-
	3	69.3	42.1	-	-
	4	77.8	47.6	-	-
	5	91.4	53.8	-	-
	6	97.1	87.0	7.8	5.6
	7	97.7	96.0	-	-
	8	97.9	97.5	8.6	4.2
I+II	11	98.1	97.8	0.0	0.0
	12	98.2	98.0	5.8	5.9
	14	98.2	98.1	8.0	7.8
	15	96.3	95.7	8.1	8.6
	19	97.6	96.8	13.9	17.3

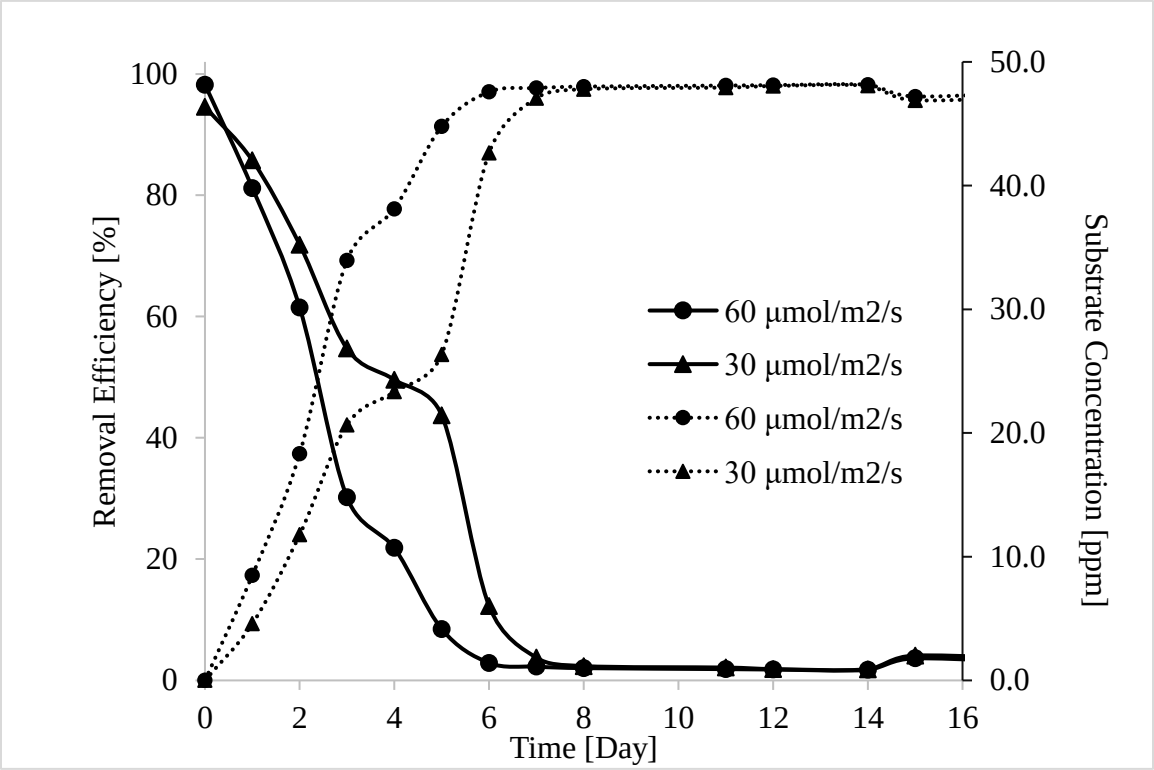


Figure 20. TN Removal and Uptake by Parachlorella Kessleri Before Nitrogen Starvation at Different Light Intensities

4.4.3 Total Lipid Analysis

As shown in Table 21 and Figure 21, the highest lipid content of 45.3% with lipid productivity of 101.9 mg/L/day were achieved in the culture under 9 days nitrogen starvation at 60 µmol·s^{-1}·m^{-2} of light intensity. Under 30 µmol·s^{-1}·m^{-2} which considered as low light, the lipid content and productivity were improved from 19.7% and 29.3 mg/L/day to 43.6% and 92 mg/L/day, respectively.

Table 21. Lipid Content, Algal Biomass and Lipid Productivity of Parachlorella Kessleri Before and After Nitrogen Starvation.

Stage	Illuminance	Algal biomass	Lipid Content	Lipid Productivity
	[µmol·s^{-1}·m^{-2}]	[g/L]	[%]	[mg/L/Day]
I	60	1.79	27.3	48.8
I+II	60	2.02	45.3	101.9
I	30	1.49	19.7	29.2
I+II	30	1.90	43.6	92.0

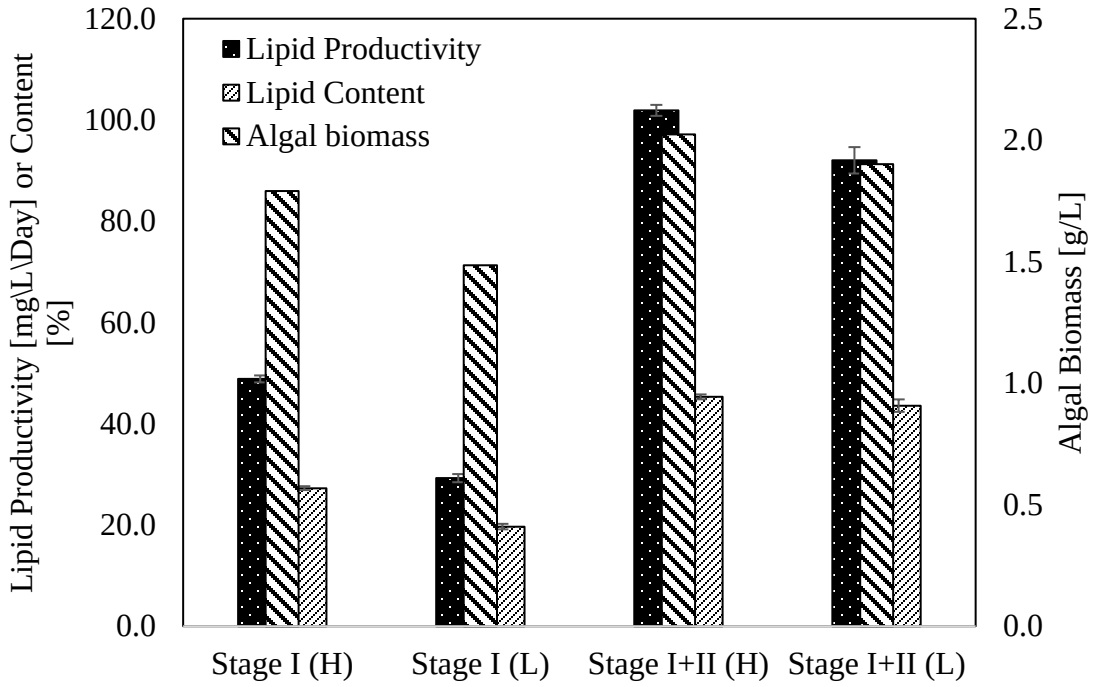


Figure 21. Lipid Content, Algal Biomass and Lipid Productivity of Parachlorella Kessleri Before and After Nitrogen Starvation.

4.5 Nitrogen to Phosphorus Ratio

4.5.1 Growth Kinetics

To study the nitrogen and phosphorus ratio effect on the cell growth and lipid accumulation of *Chlorella kessleri*, modified BBM media with 47.6, 9.5, 4.86, and 3.17 ppm of TP, respectively, were investigated at 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$. At initial TN of 47.4 ppm, the NP ratio according to the different TP concentration were 1:1, 5:1, 10:1 and 15:1, respectively. The growth curves of cell growth attained with different NP ratios are shown in Figure 22 and 22. As can be seen in Figure 24, *Chlorella kessleri* cell growth is similar for different NP ratios. However, the specific growth rate was reached the highest of 0.499 Day^{-1} at 10:1 NP ratio. On the other hand, *Parachlorella kessleri* cell growth is similar at NP ratios of 10:1 and 15:1 with specific growth rate was reached the highest of 0.452 Day^{-1}.

Table 22. Growth Characteristics of Chlorella Kessleri and Parachlorella Kessleri at Different NP Ratios.

Species	TP	μ_g	τ_D	Productivity	$NY_{X/S}$	$PY_{X/S}$	Chlorophyll
	[ppm]	[Day^{-1}]	[Day]	[mg/L/Day]	[g /g]	[g /g]	[mg/g]
CK	47.4	0.503	1.38	154.7	40.1	45.8	14.1
CK	9.48	0.453	1.53	150.5	39.2	187.1	16.1
CK	4.74	0.499	1.39	150.6	38.0	390.2	13.3
CK	3.16	0.417	1.66	142.3	36.0	595.2	13.7
PK	47.4	0.437	1.59	100.17	34.3	98.7	13.2
PK	9.48	0.440	1.58	102.01	34.6	171.4	16.8
PK	4.74	0.452	1.53	94.84	31.4	375.3	13.1
PK	3.16	0.452	1.53	98.92	34.3	674.1	12.6

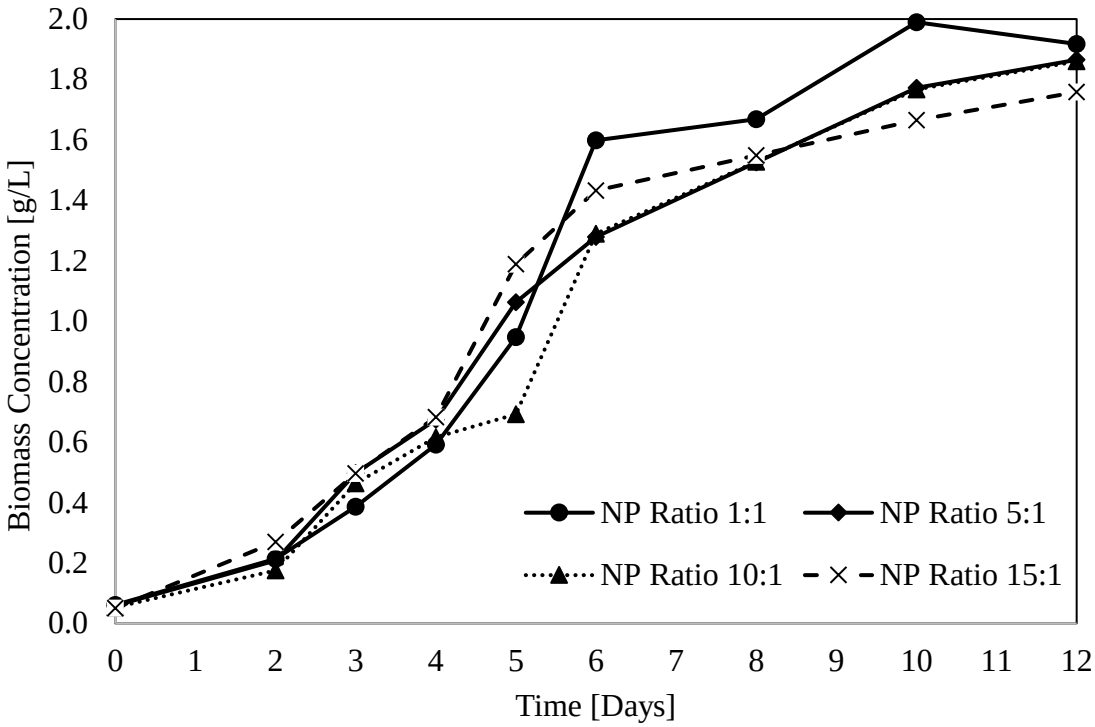


Figure 22. Growth of Chlorella Kessleri at Different NP Ratios.

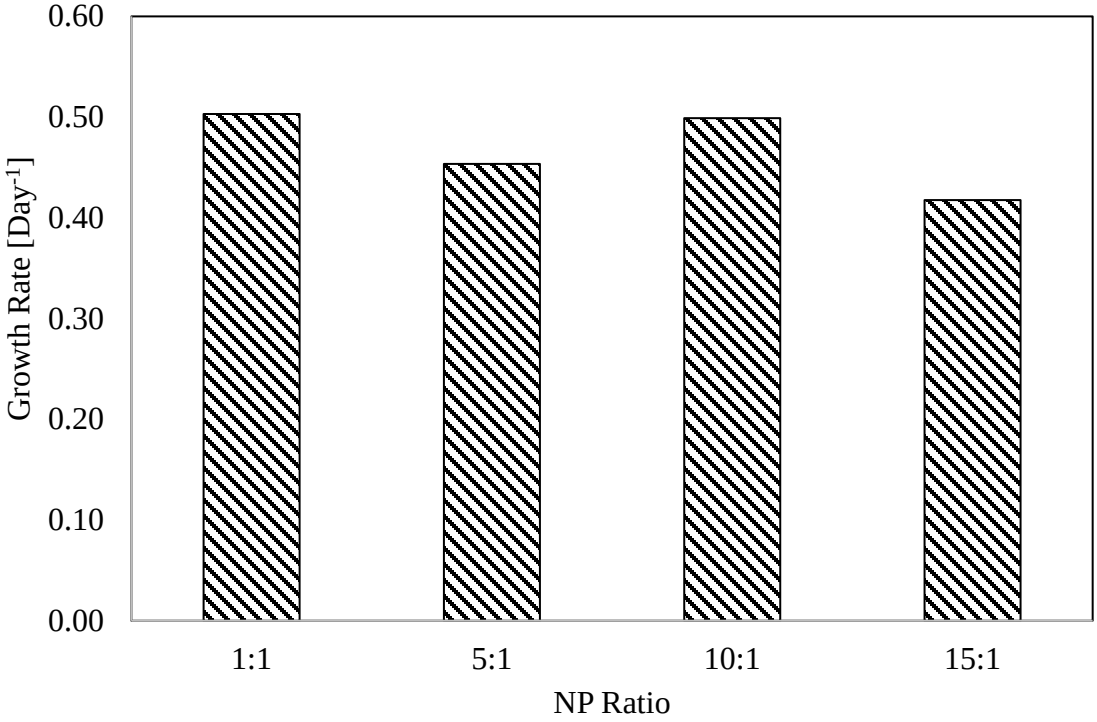


Figure 23. Growth Rate of Chlorella Kessleri at Different NP Ratios.

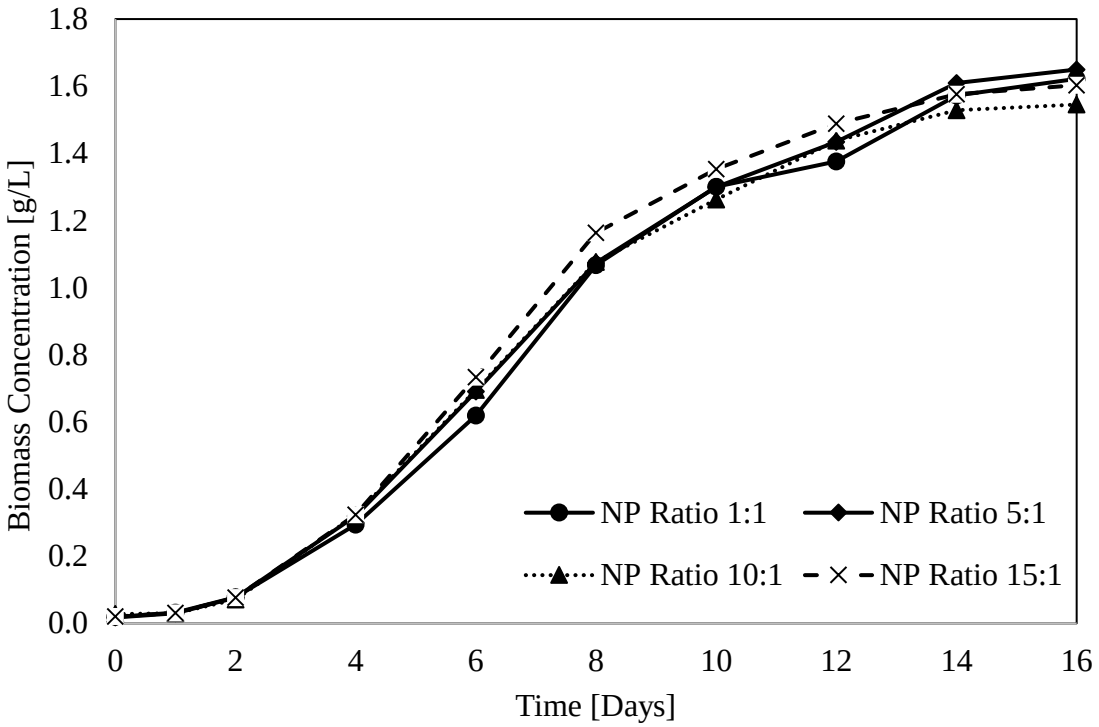


Figure 24. Growth of Parachlorella Kessleri at Different NP Ratios.

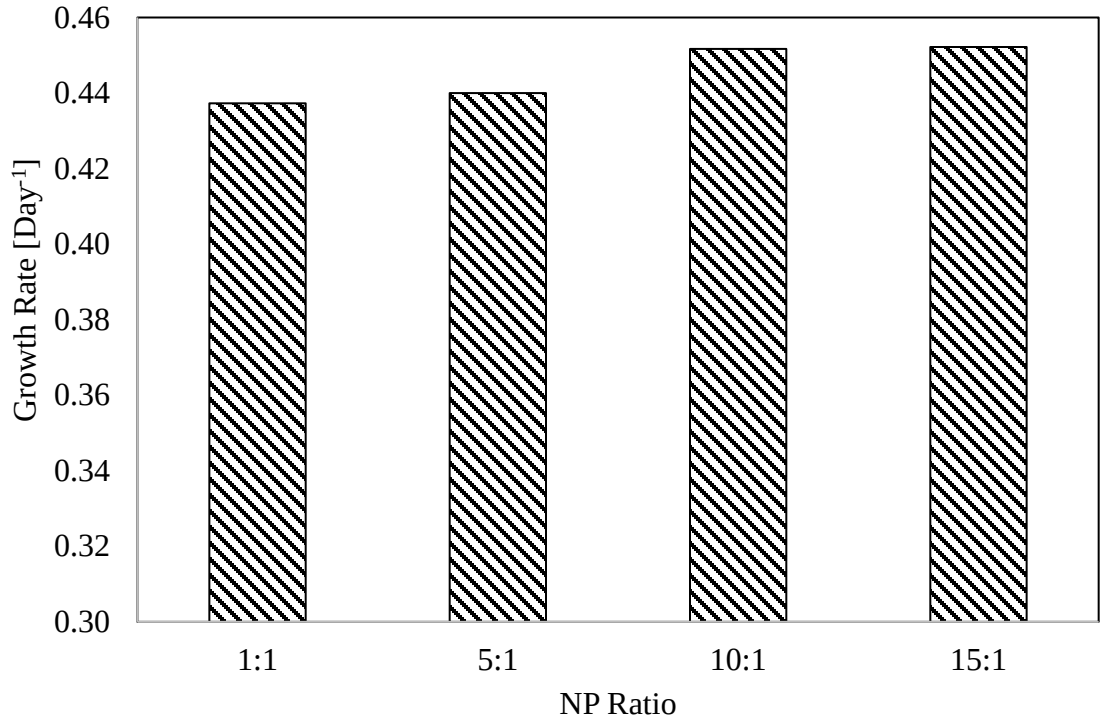


Figure 25. Growth Rate of Parachlorella Kessleri at Different NP Ratios.

4.5.2 Nutrient Uptake and Removal

The nitrogen and phosphorus removal efficiencies after twelve days cultivation of *Chlorella kessleri* and *Parachlorella kessleri* for different NP ratios are shown in Table 23 to 26. As shown in Tables, Phosphorus achieved 100% removal efficiency at N/P ratios of 5:1 to 15:1. However. the nitrogen removal was clearly not affected significantly by the NP ratios with our research conditions. Therefore, at NP ratios between 5:1 to 15:1, nitrogen and phosphorus could be both practically removed. The removal efficiency of nitrogen could be decreasing due to phosphorus limitation [40].

Table 23. TN Uptake and Removal by Chlorella Kessleri with Different NP Ratios.

Day	TN (ppm)				Removal TN (%)			
	1:1	5:1	10:1	15:1	1:1	5:1	10:1	15:1
0	47.4	47.1	48.6	48.7	0.0	0.0	0.0	0.0
2	40.1	28.5	30.5	30.8	15.4	39.5	37.3	36.7
3	28.4	12.1	10.3	14.5	40.1	74.3	78.7	70.3
4	12.9	1.9	2.3	1.3	72.7	95.9	95.3	97.3
5	8.9	1.9	0.8	0.9	81.1	96.0	98.3	98.2
6	4.8	0.8	2.5	0.7	89.9	98.4	94.9	98.5
8	1.0	0.9	1.3	0.8	97.8	98.2	97.4	98.4
10	1.5	0.8	0.9	1.2	96.9	98.2	98.1	97.6
12	1.1	1.0	1.1	1.3	97.7	97.9	97.8	97.3

Table 24. TP Uptake and Removal by Chlorella Kessleri with Different NP Ratios.

Day	TP (ppm)				Removal TP (%)			
	1:1	5:1	10:1	15:1	1:1	5:1	10:1	15:1
0	40.5	9.7	4.6	2.9	0.0	0.0	0.0	0.0
2	39.9	6.7	2.4	0.8	1.6	31.1	48.6	71.6
3	38.6	4.8	0.7	0.2	4.8	50.0	85.2	94.3
4	37.2	2.3	0.0	0.0	8.2	76.4	99.3	100.0
5	36.7	0.5	0.0	0.0	9.4	94.6	100.0	100.0
6	36.6	0.2	0.0	0.0	9.7	98.3	100.0	100.0
8	38.4	0.0	0.0	0.0	5.3	100.0	100.0	100.0
10	36.5	0.0	0.0	0.0	9.9	100.0	100.0	100.0
12	35.1	0.0	0.0	0.0	13.5	100.0	100.0	100.0

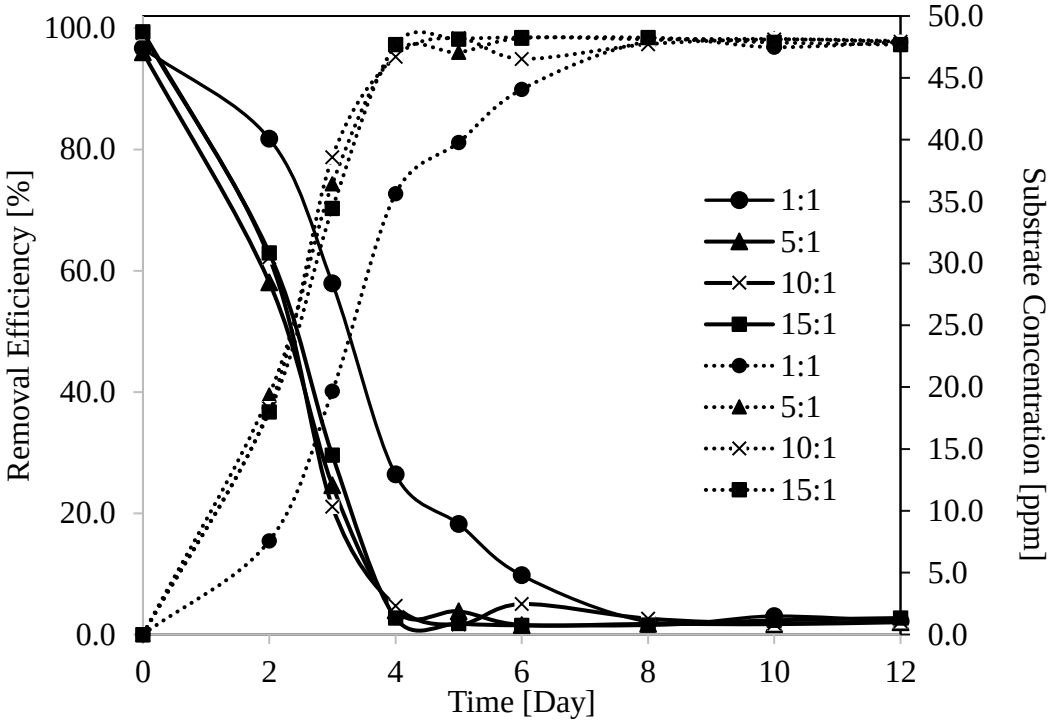


Figure 26. TN Uptake and Removal by Chlorella Kessleri with Different NP Ratios.

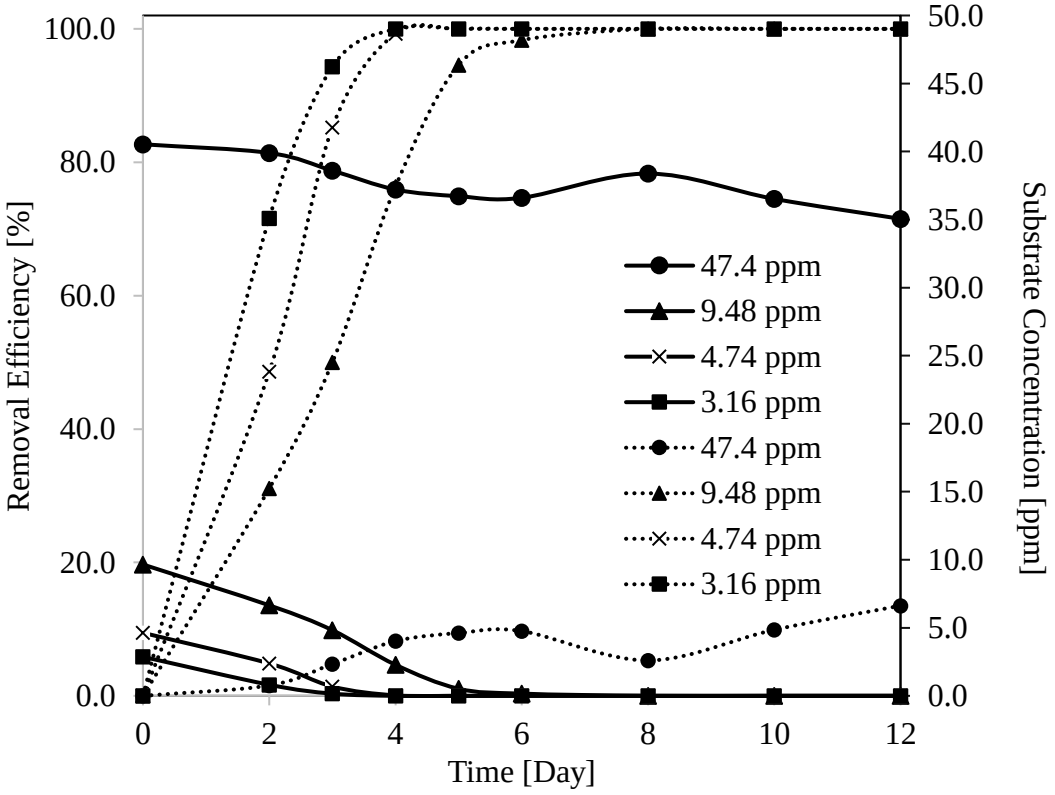


Figure 27. TP Uptake and Removal by Chlorella Kessleri with Different NP Ratios.

Table 25. TN Uptake and Removal by Parachlorella Kessleri with Different NP Ratios.

Day	TN (ppm)				Removal TN (%)			
	1:1	5:1	10:1	15:1	1:1	5:1	10:1	15:1
0	46.7	47.2	48.3	46.1	0.0	0.0	0.0	0.0
1	44.5	45.8	45.1	45.7	4.6	2.9	6.7	0.9
2	34.0	38.0	34.8	36.0	27.2	19.4	28.0	21.9
4	19.1	16.7	16.4	14.6	59.0	64.7	66.1	68.4
6	3.3	4.9	5.2	1.2	93.0	89.6	89.2	97.4
8	0.6	1.0	1.2	1.3	98.6	97.9	97.4	97.2
10	0.2	0.3	0.4	0.4	99.5	99.3	99.1	99.2
12	0.0	0.0	0.0	0.0	100.0	100.0	100.0	100.0
14	0.0	0.0	0.0	0.0	100.0	100.0	100.0	100.0
16	0.0	0.0	0.0	0.0	100.0	100.0	100.0	100.0

Table 26. TP Uptake and Removal by Parachlorella Kessleri with Different NP Ratios.

Day	TP (ppm)				Removal TP (%)			
	1:1	5:1	10:1	15:1	1:1	5:1	10:1	15:1
0	49.9	9.5	4.0	2.3	0.0	0.0	0.0	0.0
1	49.5	9.1	4.0	2.1	0.8	4.4	1.6	12.5
2	45.3	7.8	2.6	1.0	9.3	18.5	35.5	55.6
4	40.4	5.0	0.9	0.3	19.0	47.6	76.6	88.2
6	38.2	2.6	0.1	0.0	23.4	72.3	97.2	98.6
8	35.7	1.7	0.0	0.0	28.5	82.5	100.0	100.0
10	34.8	0.6	0.0	0.0	30.3	93.7	100.0	100.0
12	34.9	0.1	0.0	0.0	30.1	98.6	100.0	100.0
14	34.4	0.0	0.0	0.0	31.0	100.0	100.0	100.0
16	33.7	0.0	0.0	0.0	32.5	100.0	100.0	100.0

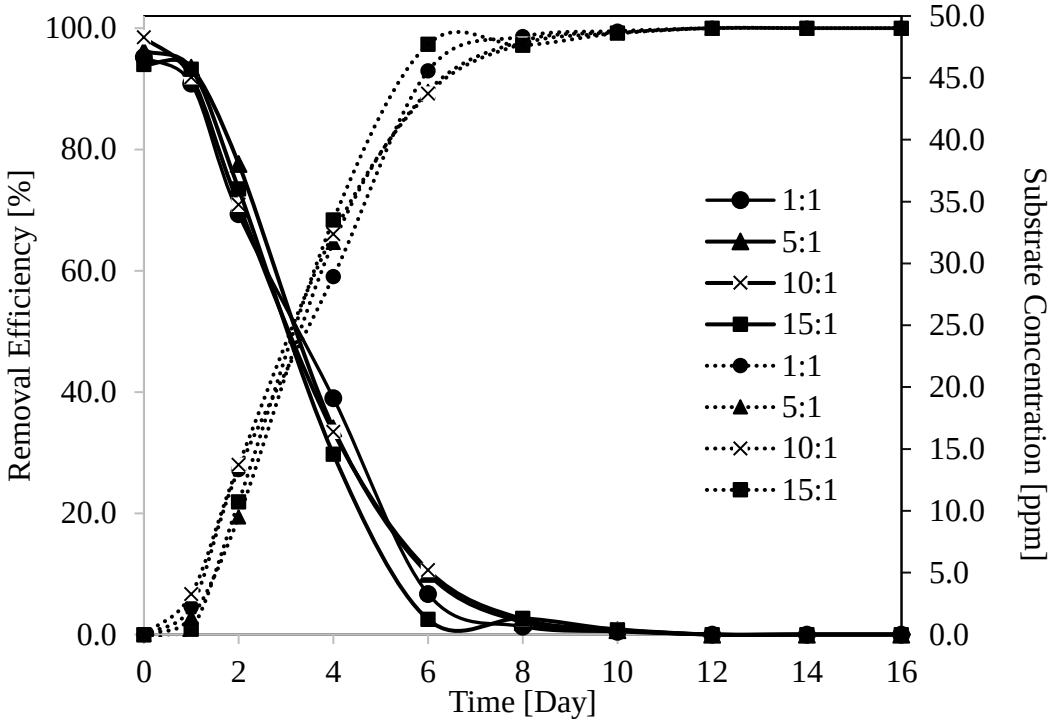


Figure 28. TN Uptake and Removal by Parachlorella Kessleri with Different NP Ratios.

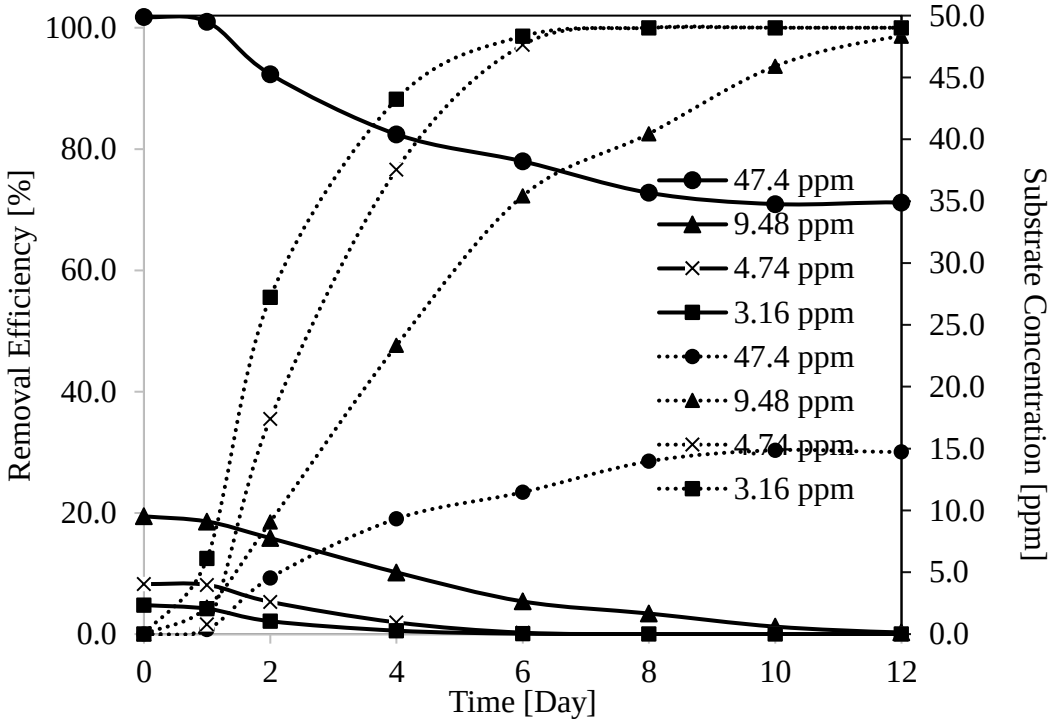


Figure 29. TP Uptake and Removal by Parachlorella Kessleri with Different NP Ratios.

4.5.3 Total Lipid Analysis

The total lipid growth of *Chlorella kessleri* under different NP ratios are shown in Table 27, Figure 30 and 31. As shown in Figure 30 and 31, at an initial TP of 47.6 ppm, the total lipid content was around 27.3% and 37.5, respectively. Nevertheless, when *Chlorella kessleri* and *Parachlorella kessleri* were investigated at different NP ratios in the condition of phosphorus limitation, the growth of total lipid was enhanced with total lipid content between 39.4 to 45.0%. Moreover, the lipid productivity reached as high as 64.0 mg/L/day at 5:1 and 15:1 NP ratios. Nutrient limitation has a significant effect to improve the lipid content per algal biomass, and it has been testified by many researchers. Rodolfi et al. found that the lipid content of *Nannochloropsis sp.* improved from 32% up to 60% when the culture condition transferred from nitrogen sufficient to nitrogen deprived [74]. Kapdan and Aslan reported that the NP ratio had a significant effect on the removal of nutrients and found that the optimal NP ratio for *Chlorella vulgaris* was 8:1 [132]. Goldberg and Cohen stated that under phosphorus limitation condition, the total lipid content of starved cells improved owing to the substantial increase in triacyl glyceride (TAG) content from 6.5% to 39.3% [59]. In addition, Courchesne et al. stated that when the photosynthesis cellular mechanisms are active in presence of light and CO_2, microalgae accumulate lipids under nutrient limitation conditions [133]. Li et al. reported that high removal efficiencies for both nitrogen and phosphorus can be achieved with controlled NP ratio between 5:1 to 8:1, though, during the nitrogen or phosphorus limitation, the highest total lipid content of *Scenedesmus sp.* was contradicted with lower lipid productivity rate due to the comparatively low biomass of microalgae [40]. In this study at lower initial concentrations of phosphorus, *Chlorella kessleri* and *Parachlorella kessleri* produced the highest total lipid

content of 45.0% and 43.9%, respectively, accompanying with highest lipid productivity rate of 64.0 and 43.4 mg/L/day, respectively, at NP ratio of 15:1 with aeration with 1.5% CO_2, and initial TN and TP concentrations of 47.4 and 3.17 ppm, respectively.

Table 27. Lipid Content, Algal Biomass and Lipid Productivity of Chlorella Kessleri and Parachlorella Kessleri at Different NP Ratios.

TP	Species	Algal biomass	Lipid Content	Lipid Productivity
[ppm]	[$\mu mol \cdot s^{-1} \cdot m^{-2}$]	[g/L]	[%]	[mg/L/Day]
47.4	CK	1.86	27.3	42.2
9.48	CK	1.81	42.3	63.7
4.74	CK	1.81	39.4	59.3
3.16	CK	1.71	45.0	64.0
47.4	PK	1.60	37.5	37.6
9.48	PK	1.63	35.8	36.5
4.74	PK	1.52	44.6	42.3
3.16	PK	1.58	43.9	43.4

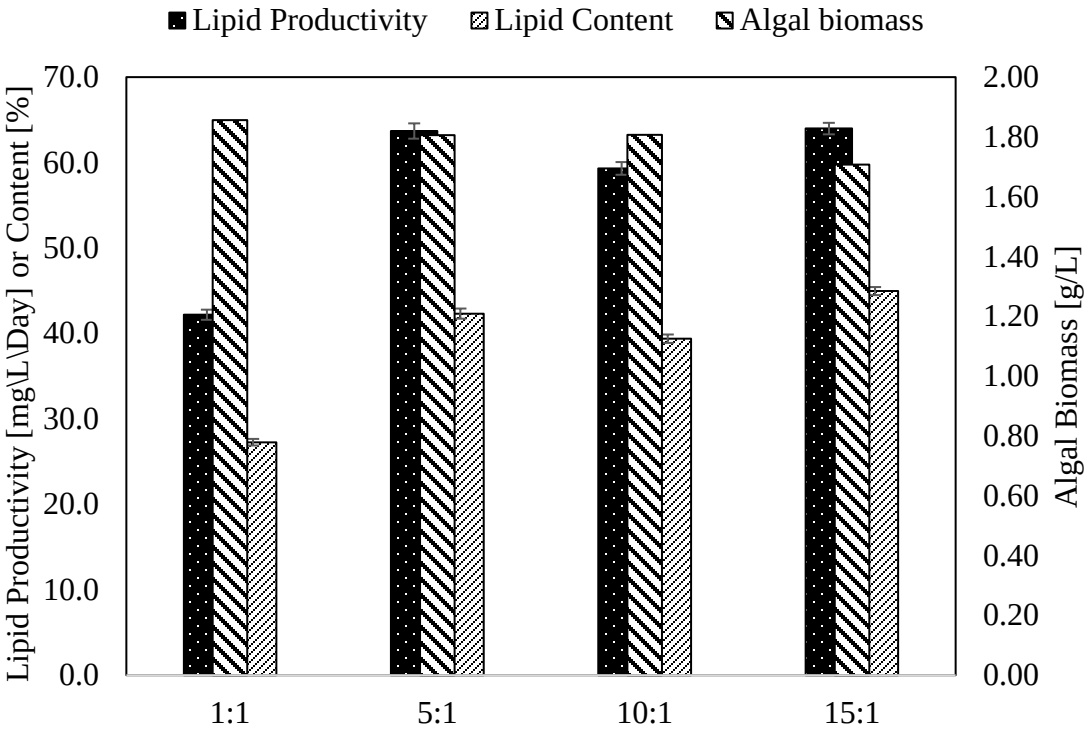


Figure 30. Lipid Content, Algal Biomass and Lipid Productivity of Chlorella Kessleri at Different NP Ratios.

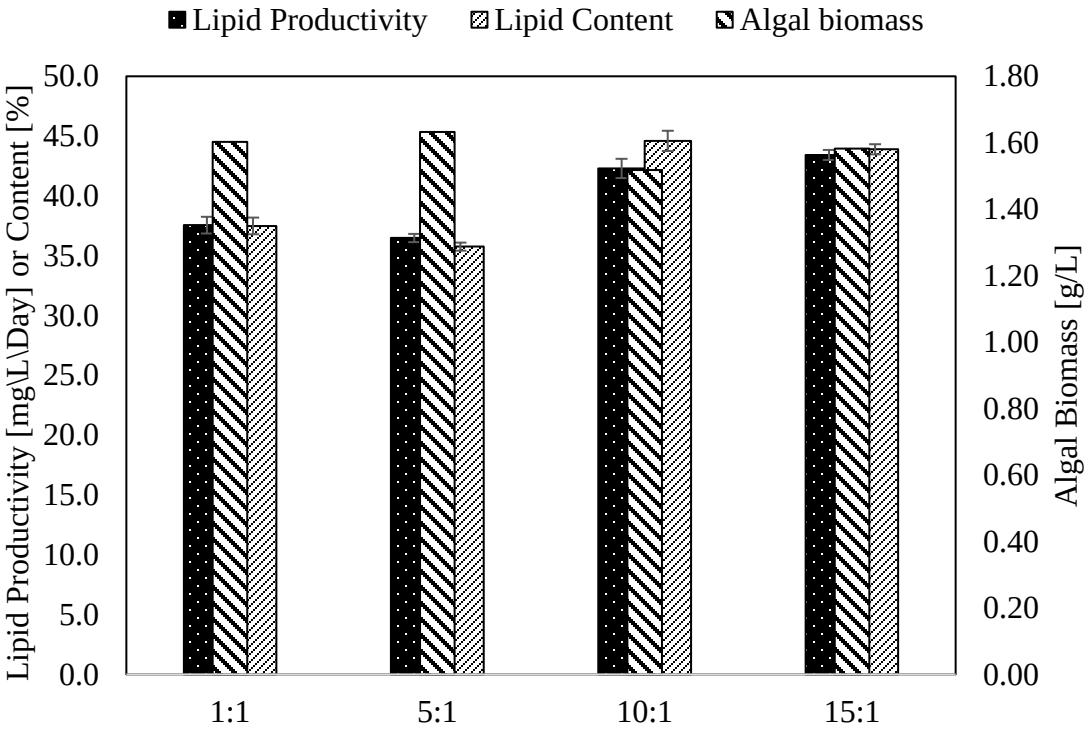


Figure 31. Lipid Content, Algal Biomass and Lipid Productivity of Parachlorella Kessleri at Different NP Ratios.

4.6 CO_2 Capture and Light Intensity

4.6.1 Growth Kinetics

Parachlorella kessleri were cultivated at 25 ± 1 °C at different light intensities of 100, 60 and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in modified BBM medium under various CO_2 concentrations. The initial inoculum concentration was 0.017 $g \cdot L^{-1}$ and the initial pH was around 7.9 ± 0.68. The CO_2 concentrations effects on the microalgal growth of *Parachlorella kessleri* at different light intensities are shown in Figure 32, 33 and 34. Earlier studies stated that exceeding the CO_2 concentration with aeration above 5% might harm the algal cells and inhibit their growth [76,78,84]. In our present work, *Parachlorella kessleri* could grow well under the relatively high CO_2 concentrations between 6% to 15% as can be seen in Figure 29, 30 and 31. Tang et al. [134] reported the maximum biomass concentration of C. pyrenoidosa SJTU-2 was about 1.55 $g \cdot L^{-1}$ at 10% of CO_2 concentration after 14 days cultivation. The maximum biomass concentration of *Parachlorella kessleri* under CO_2 concentration of 10% after 14 days cultivation was 1.97 $g \cdot L^{-1}$ as shown in Table 28, which was almost 1.3 times higher than that of *C. pyrenoidosa*. Chiu et al. [77] investigated *N. oculata* and *Chlorella sp.* that achieved an optimal growth potential with CO_2 concentration of 2%, while algal growth inhibition reported under CO_2 concentration of 5, 10, and 15%. However, the maximum biomass concentration attained was around 1.2 $g \cdot L^{-1}$ of *N. oculata* and *Chlorella sp.* under 2% CO_2. In our study, we attained the maximum biomass concentration under 2% CO_2 concentration with 2.09 $g \cdot L^{-1}$ which was almost 1.7 times higher than that of *N. oculata* and *Chlorella sp.* As can be seen in Table 28 and 30, locally isolated *Parachlorella kessleri* showed higher biomass concentration, productivity and

specific growth rate under the CO_2 concentrations ranging from 4% to 15%. The maximum biomass concentration of 2.09 and 1.97 $g \cdot L^{-1}$, maximum biomass productivity of 0.164 $g \cdot L^{-1} \cdot Day^{-1}$ at 2 and 10% CO_2 concentrations, respectively, and maximum specific growth rate of 1.204 Day^{-1} at 15% CO_2 concentration all at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Table 28. Growth Characteristics of Parachlorella Kessleri at Different Light Intensities and CO_2 Concentrations.

CO2	Illuminance	μ_g	τ_D	Productivity	$Y_{X/S}$	Chlorophyll
%	[$\mu mol \cdot s^{-1} \cdot m^{-2}$]	[Day^{-1}]	[Day]	[mg/L/Day]	[g /g]	[mg/g]
0.0	30	0.252	2.75	117.3	29.7	17.5
2.0	30	0.384	1.80	132.6	33.6	14.5
4.0	30	0.350	1.98	138.7	35.1	22.4
6.0	30	0.357	1.94	135.8	34.4	18.9
10.0	30	0.478	1.45	124.8	31.6	21.6
15.0	30	0.512	1.36	123.0	31.1	21.7
0.0	60	0.301	2.30	122.4	31.0	11.4
2.0	60	0.538	1.29	148.6	37.6	12.4
4.0	60	0.467	1.48	148.1	37.5	22.8
6.0	60	0.469	1.48	159.2	40.3	16.9
10.0	60	0.508	1.36	137.3	34.7	18.0
15.0	60	0.527	1.31	130.3	33.0	19.0
0.0	100	0.418	1.66	105.7	26.8	4.9
2.0	100	0.674	1.03	164.7	41.7	3.1
4.0	100	0.522	1.33	157.6	39.9	4.4
6.0	100	0.717	0.97	160.6	40.7	9.8
10.0	100	1.143	0.61	164.0	41.5	5.7
15.0	100	1.204	0.58	151.7	38.4	4.2

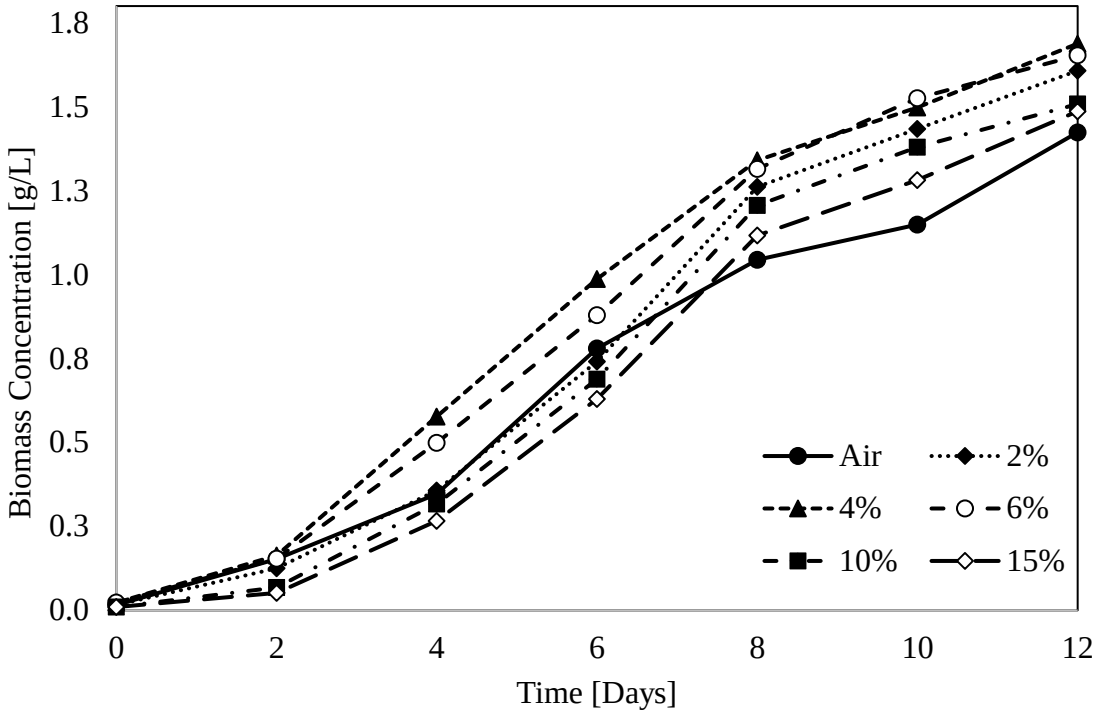


Figure 32. Growth of Parachlorella Kessleri at 30 µmol·s^{-1}·m^{-2} with Different CO_2 Concentrations.

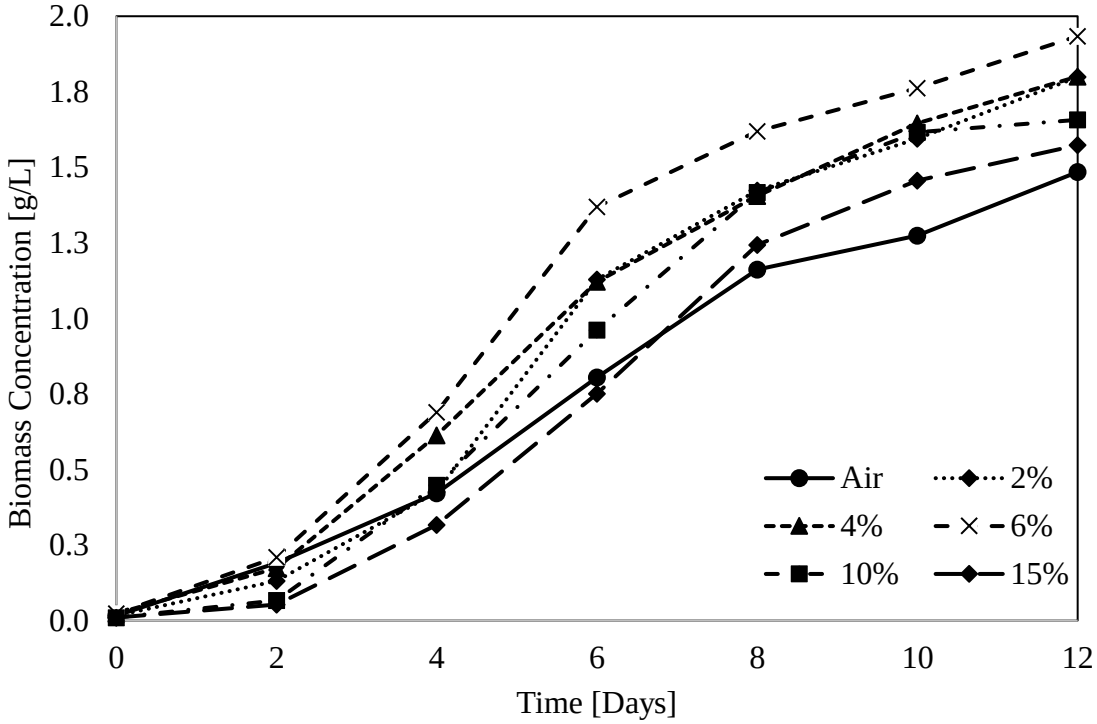


Figure 33. Growth of Parachlorella Kessleri at 60 µmol·s^{-1}·m^{-2} with Different CO_2 Concentrations.

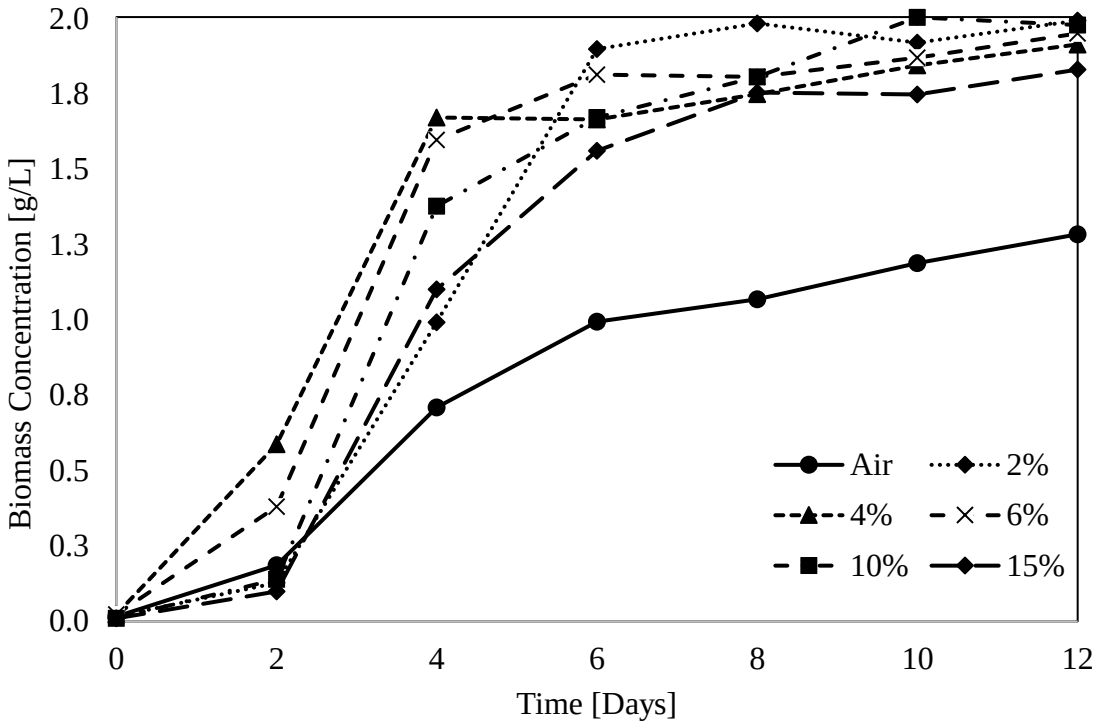


Figure 34. Growth of Parachlorella Kessleri at 100 µmol·s^{-1}·m^{-2} with Different CO_2 Concentrations.

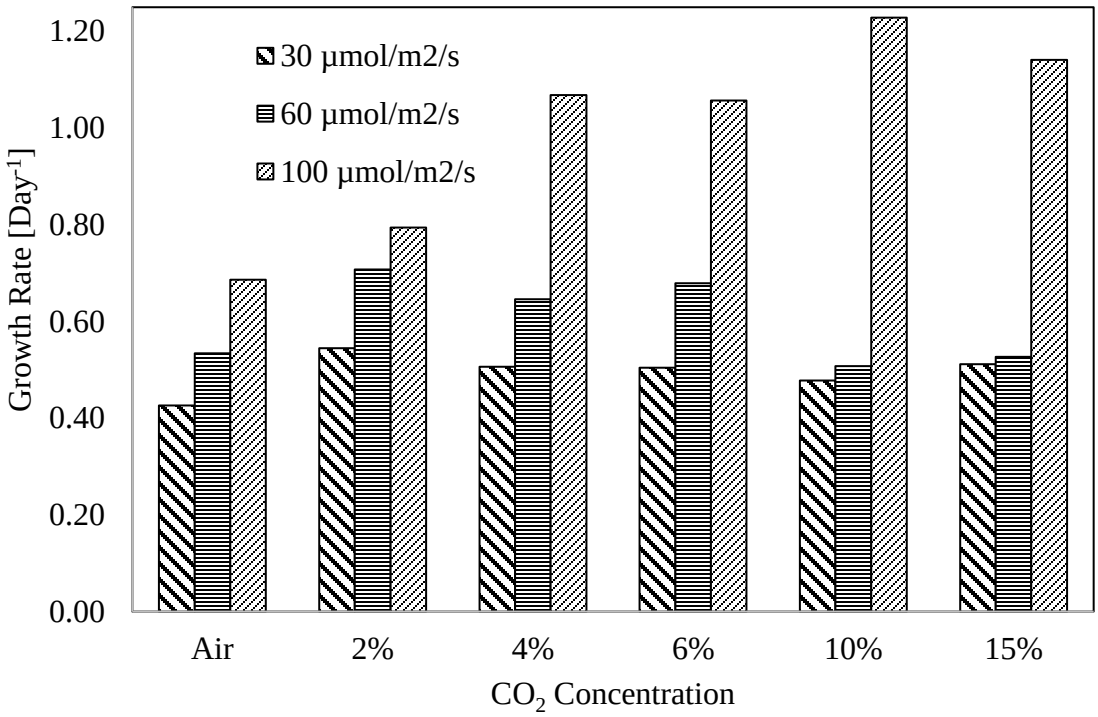


Figure 35. Growth Rate of Parachlorella Kessleri at Different CO_2 Concentrations and Light Intensities.

4.6.2 pH Profile

In the bubble system, it is presumed that the pH profiles attained with the pH probe are the equivalent to the bulk culture of the microalgae media, and the algal culture is well-mixed. Despite the same extracellular pH, the intracellular pH can be affected by the extracellular CO2 equilibrium concentration, whereas higher extracellular equilibrium CO_2 concentration leads to a lower intracellular pH which might inhibit the enzymes involved in photosynthesis. In *Parachlorella kessleri*, fixed CO_2 by Rubisco during photosynthesis is taken from the bulk medium in form of HCO_3 by inorganic carbon transporters to the chloroplasts conferring to the following equilibrium equation:

$$H^+ + HCO_3^- \Leftrightarrow CO_2 + H_2O$$

Where H^+ is consumed during the conversion of HCO_3 to CO_2. Therefore, steady-state usage of HCO_3 leaves OH^- in the cell which can be neutralized by H^+ uptake from unfixed carbon in the extracellular environment. H^+ reduction of the medium inevitably increases pH. As can be seen in Figures 36, pH starts at 7.0 to 8.0 then dropped so fast during the lag phase due to unfixed carbon and nutrients uptake to adapt to the environment. In growth phase, algal cells start to consume the nitrogen and that increases the pH according to the nitrate assimilation by Kirkby and Knight [135]:

$$NO_3^- + 8H^+ + 8e^- \Leftrightarrow NH_3 + 2H_2O + OH^-$$

As light increases more fixation happens and more uptake achieved, therefore, pH is increasing until it reaches stationary phase then its became naturalized by the unfixed carbon to sustain the algal culture.

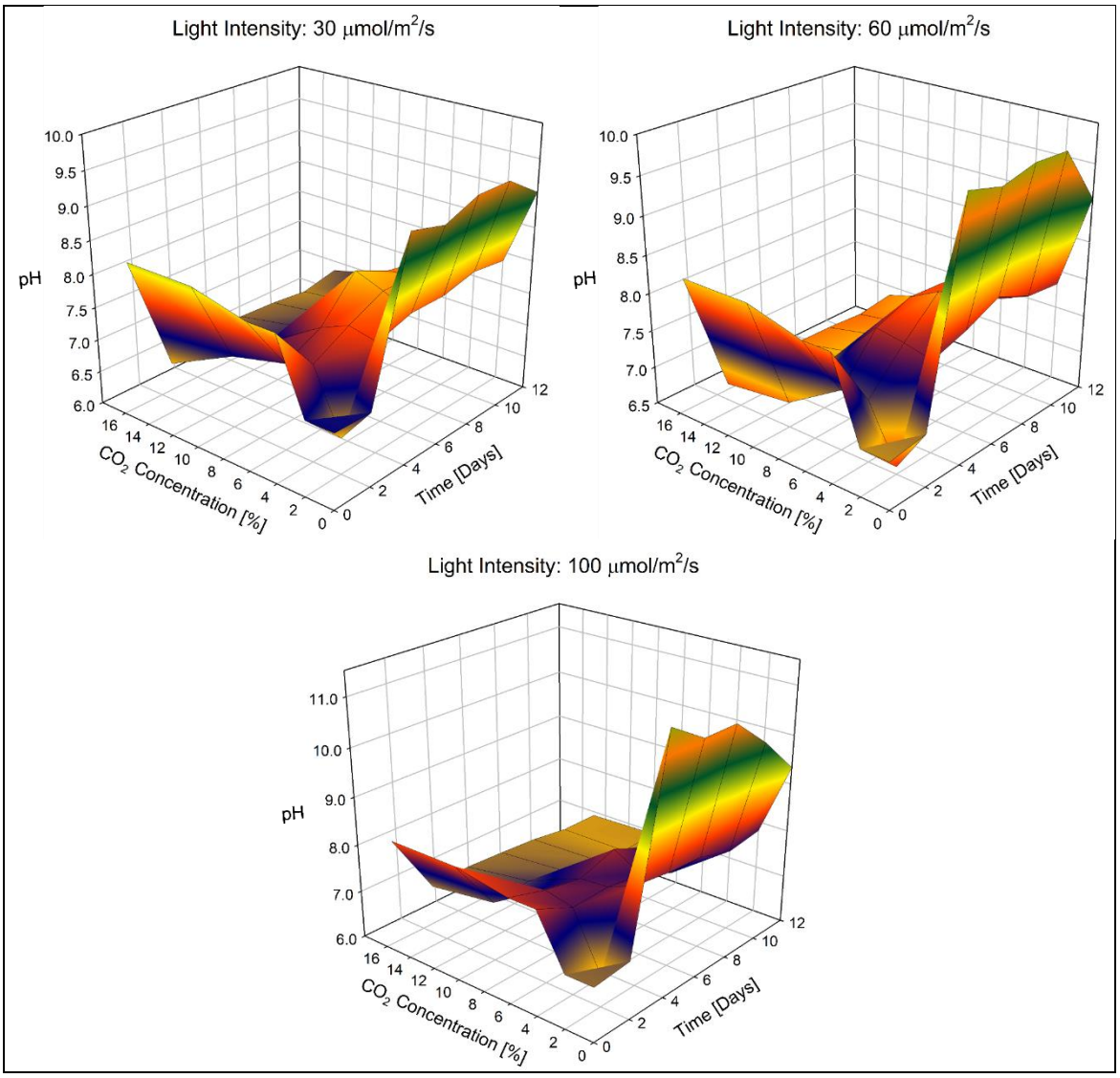


Figure 36. pH Profile of Parachlorella Kessleri Culture at Different Light Intensities with Different CO2 Concentrations.

4.6.3 Elemental Analysis and CO_2 Fixation

Carbon dioxide uptake rate can be determined by the microalgal biomass productivity assuming that all the CO_2 assimilated was converted into biomass. Average fixed carbon content was measured by the CHNSO elemental analyzer. Table 29 shows CO_2 fixation rates calculated multiplying the biomass productivity with the measured carbon content equivalent CO_2 in the different supply of CO_2 concentration studied coupled with the effect of light intensity on this parameter. For most algal strains, higher light intensities resulted in higher CO_2 uptake rate due to the increase in cells growth and biomass productivities. Since used light intensities are below the light saturation point, then photosynthetic rate is directly proportionally to light irradiance that resulted in an increase in biomass productivities and CO_2 fixation rate. Photoautotrophic mode was considered which means exposure time to light is maximum and that increase the CO_2 fixation rates as observed by Gonçalves et al. [136], Jacob-Lopes et al. [137] and Pires et al. [138]. A maximum value of 0.363 $g \cdot L^{-1} \cdot Day^{-1}$ was obtained for *Parachlorella kessleri* grown with light intensity of 100 $\mu mol \cdot s^{-1} \cdot m^{-2}$ under 10% CO_2 concentration. As can be seen in Figure 36 that locally isolated *Parachlorella kessleri* shows a potential to grow under high CO_2 concentration and can be effective in CO_2 capture from the atmosphere due to its high biomass productivities.

Table 29. Elemental Compositions and CO_2 Fixation Rate of Parachlorella Kessleri Culture with Different CO_2 Concentrations and Light Intensities.

CO2	Illuminance	C	H	N	Productivity	CO_2 Fixation Rate
%	[$\mu mol \cdot s^{-1} \cdot m^{-2}$]	[%]	[%]	[%]	[mg/L/Day]	[g/L/Day]
0.0	30	42.73	6.48	3.62	117.3	0.184
2.0	30	47.70	7.57	3.04	132.6	0.232
4.0	30	52.57	9.21	3.37	138.7	0.267
6.0	30	54.70	9.73	3.39	135.8	0.272
10.0	30	53.52	7.32	3.59	124.8	0.245
15.0	30	53.60	9.38	3.58	123.0	0.242
0.0	60	50.04	8.73	3.43	122.4	0.224
2.0	60	52.05	9.74	2.75	148.6	0.283
4.0	60	53.44	9.41	3.11	148.1	0.290
6.0	60	54.09	9.65	2.74	159.2	0.316
10.0	60	55.03	9.41	3.08	137.3	0.277
15.0	60	55.54	9.21	3.35	130.3	0.265
0.0	100	48.73	8.62	2.68	105.7	0.189
2.0	100	52.61	10.41	1.55	164.7	0.318
4.0	100	55.20	12.01	2.07	157.6	0.319
6.0	100	57.16	11.43	2.05	160.6	0.336
10.0	100	60.35	11.18	1.85	164.0	0.363
15.0	100	61.35	10.78	2.14	151.7	0.341

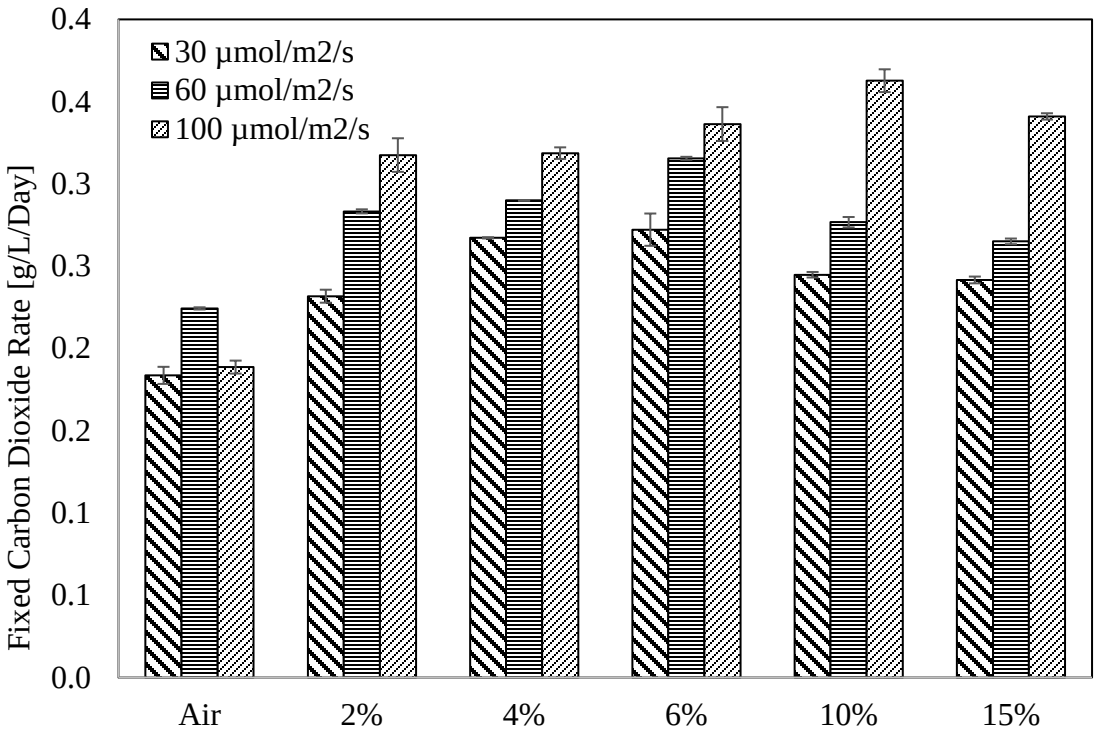


Figure 37. CO_2 Fixation Rates of Parachlorella Kessleri Culture under Different CO_2 Concentrations and Light Intensities.

4.6.4 Total Lipid Analysis

As shown in Table 30 and Figure 38 to 40, the highest lipid content of 59.9% with lipid productivity of 98.3 $mg \cdot L^{-1} \cdot Day^{-1}$ were achieved in the culture under 10% CO_2 concentration and 100 $\mu mol \cdot s^{-1} \cdot m^{-2}$ of light intensity. Also, when light intensity increases, high growth of algal biomass produced, and then high lipid productivity achieved. High light intensity and CO_2 concentration can provide more efficient photosynthesis and lipid content as more carbon is being captured.

Table 30. Algal Biomass, Lipid Productivity, Lipid, Carbs. and Protein Contents of Parachlorella Kessleri at Different CO_2 Concentrations and Light Intensities.

CO_2	Illuminance	Algal biomass	Lipid Content	Lipid Productivity	Protein Content	Carbs. Content*
%	[$\mu mol \cdot s^{-1} \cdot m^{-2}$]	[g/L]	[%]	[mg/L/Day]	[%]	[%]
0.0	30	1.46	37.9	42.5	21.5	38.7
2.0	30	1.66	41.5	53.0	18.3	38.8
4.0	30	1.62	32.4	43.8	21.3	43.9
6.0	30	1.59	29.5	39.1	20.0	48.4
10.0	30	1.50	25.9	32.3	21.4	50.5
15.0	30	1.48	32.9	40.5	21.3	43.5
0.0	60	1.53	24.9	29.2	20.4	53.6
2.0	60	1.88	26.9	38.8	16.3	55.4
4.0	60	1.74	33.0	47.8	16.3	48.3
6.0	60	1.88	34.0	53.2	18.5	45.7
10.0	60	1.65	37.8	51.9	18.3	42.0
15.0	60	1.56	33.9	44.1	19.9	44.3
0.0	100	1.30	32.0	32.0	16.2	51.3
2.0	100	2.09	45.1	72.6	9.2	45.3
4.0	100	1.86	47.6	73.6	12.2	39.8
6.0	100	1.89	47.7	75.3	12.9	38.3
10.0	100	1.97	59.9	98.3	11.0	28.4
15.0	100	1.82	55.7	84.5	12.7	31.1

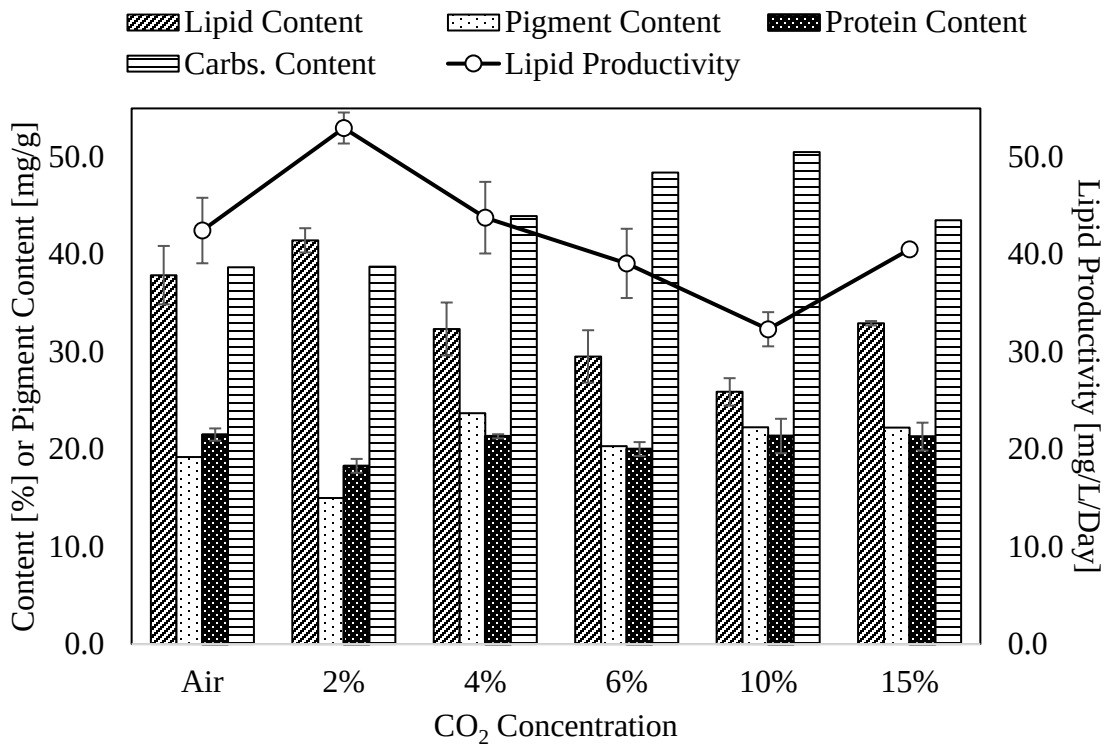


Figure 38. Algal Biomass, Lipid Productivity, Lipid, Carbs. and Protein Contents of Parachlorella Kessleri at Different CO_2 Concentrations and 30 µmol·s^{-1}·m^{-2}.

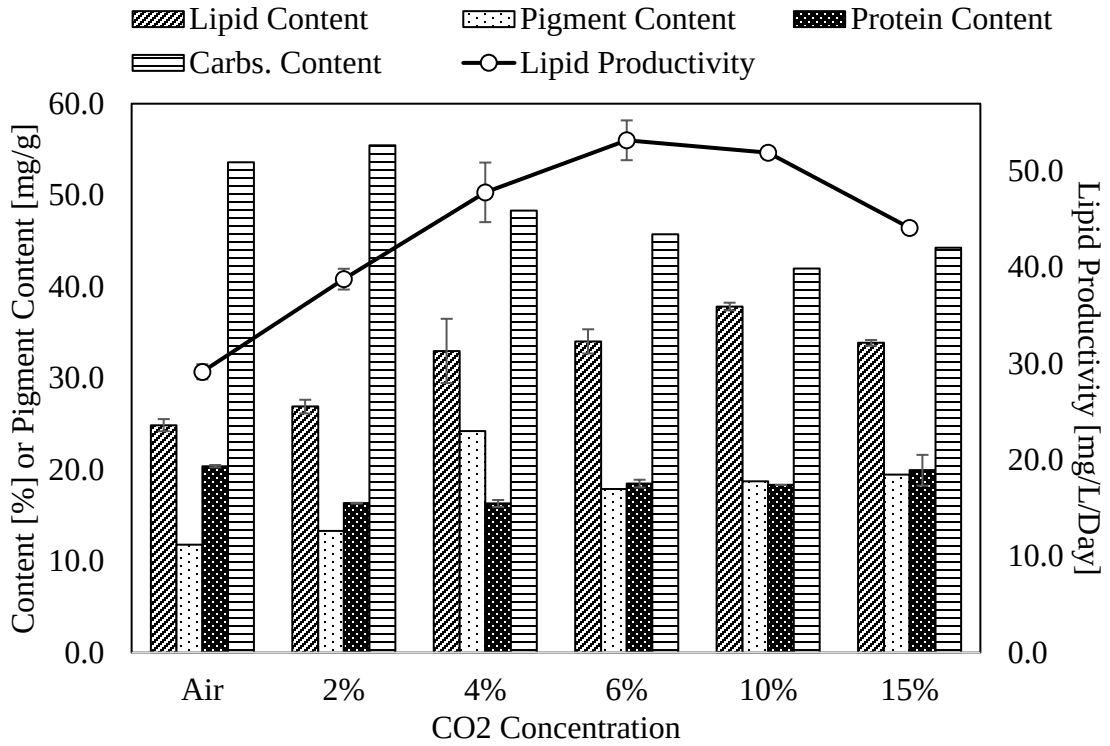


Figure 39. Algal Biomass, Lipid Productivity, Lipid, Carbs. and Protein Contents of Parachlorella Kessleri at Different CO_2 Concentrations and 60 µmol·s^{-1}·m^{-2}.

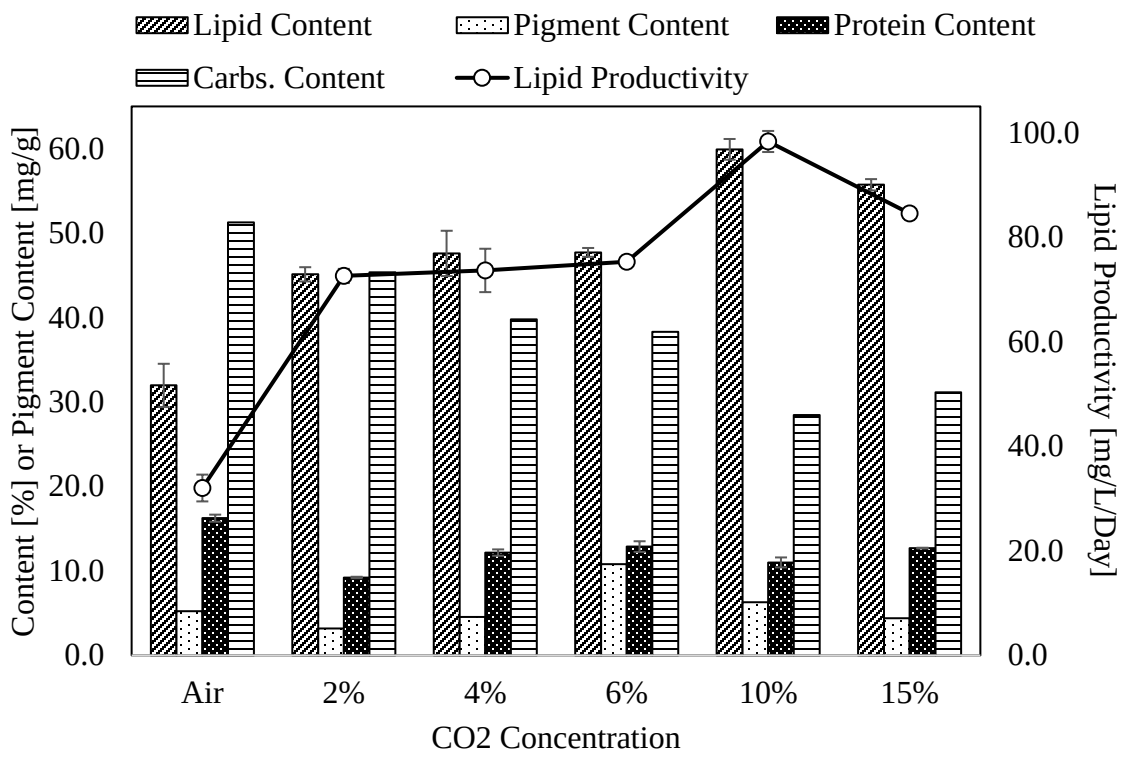


Figure 40. Algal Biomass, Lipid Productivity, Lipid, Carbs. and Protein Contents of Parachlorella Kessleri at Different CO_2 Concentrations and 100 µmol·s^{-1}·m^{-2}.

4.7 FAME Analysis

The FAME composition of the biodiesels produced by the direct transesterification of *Parachlorella kessleri* is represented in Figure 41 and 42. *Parachlorella kessleri* oil mainly contained C16 and C18 that represent around 97% of the total FAME content. As can be seen, increasing light intensity enhance the production of C16 and C18 fatty acid methyl esters.

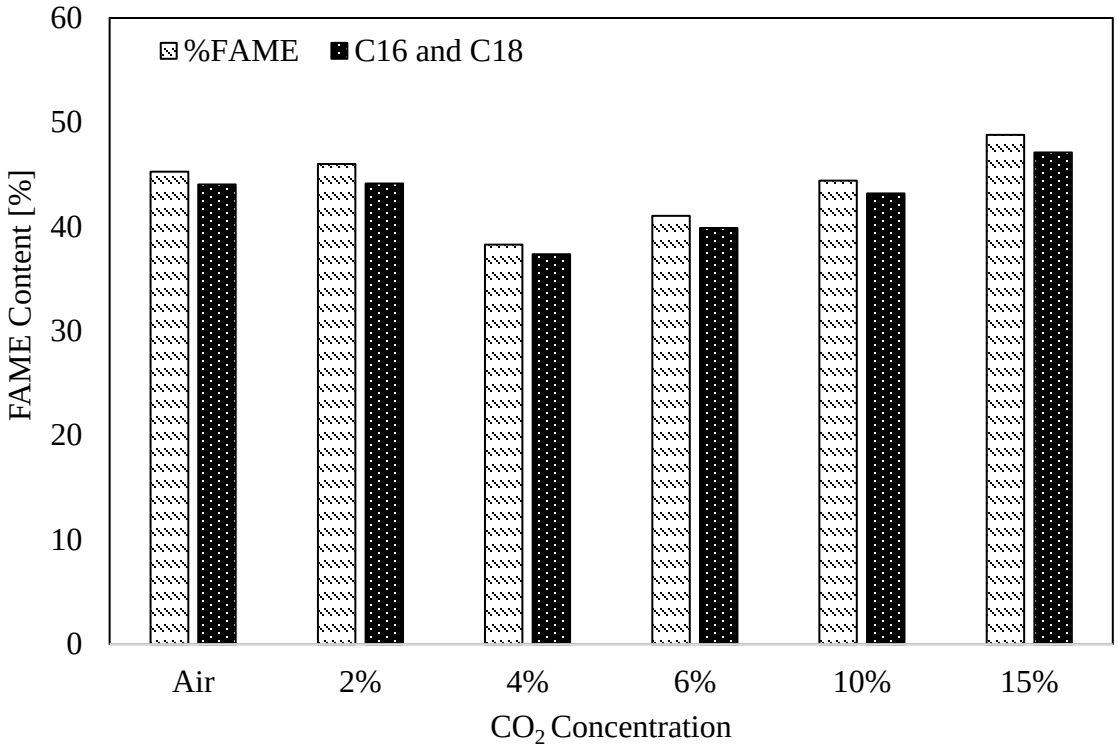


Figure 41. FAME Content & Composition of Parachlorella Kessleri at Different CO_2 Concentrations and 60 µmol·m^{-2} ·s^{-1}.

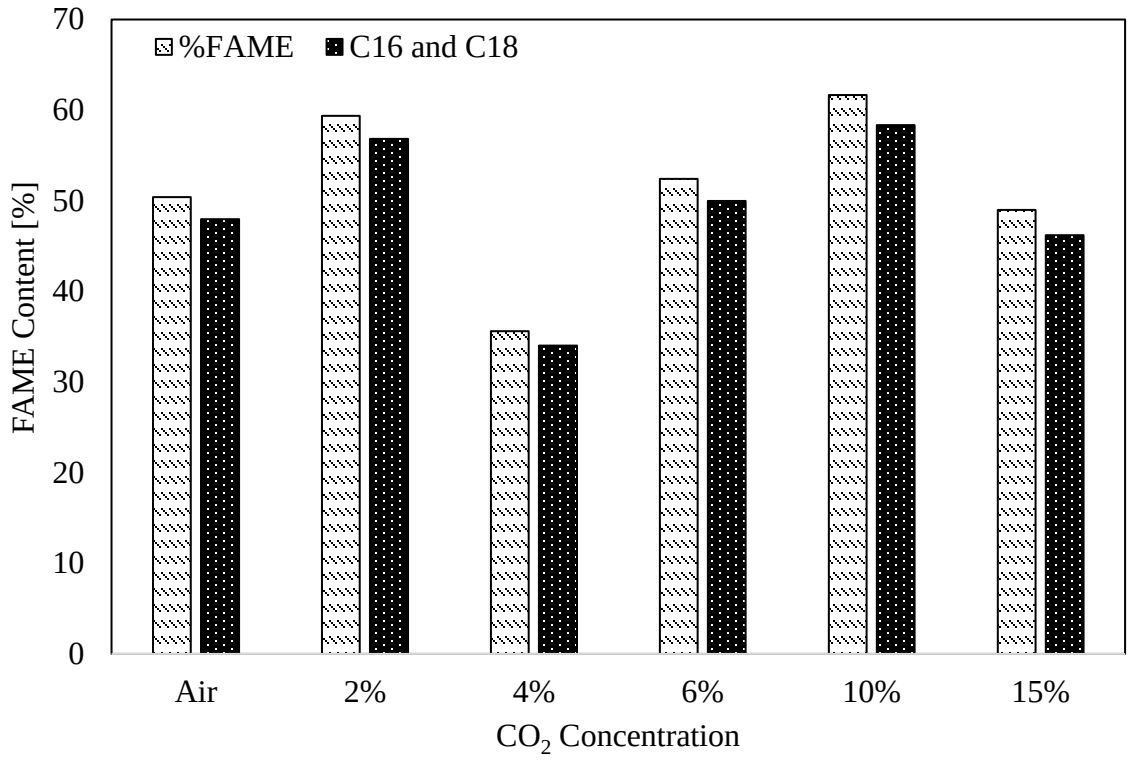


Figure 42. FAME Content & Composition of Parachlorella Kessleri at Different CO_2 Concentrations and 100 µmol·m^{-2}·s^{-1}.

4.8 Extraction Methods.

Two methods of lipid extraction were investigated at three different temperatures to evaluate which method can provide high FAME yield. As can be seen in Figure 42, as temperature increases, higher lipid and FAME content are being extracted and trans-esterified, respectively. However, Bligh and Dyer method that uses chloroform and methanol showed more efficient extraction by an average of 18.9% FAME content, in comparison with Bagoong method that uses methanol, DMSO, hexane and diethyl ether.

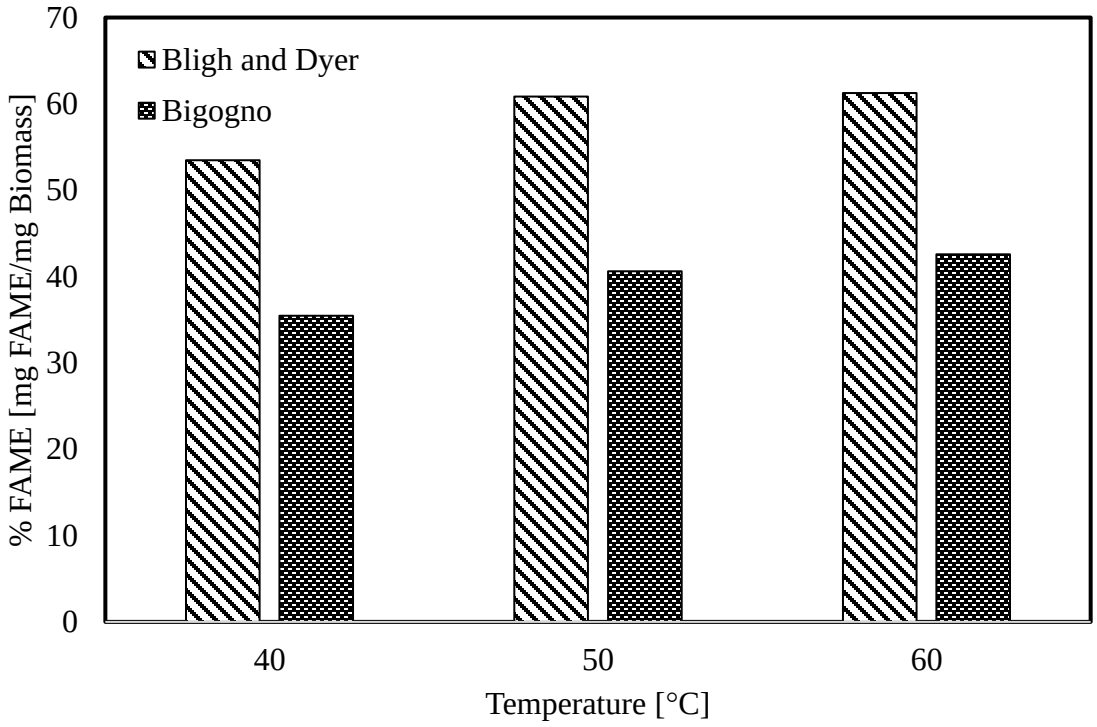


Figure 43. %FAME Using Two Extraction Methods at Different Temperatures.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

To sum up, isolated *Parachlorella kessleri* has a promising potential to capture CO2 and produce high lipid content to be converted to high value chemicals or biodiesel. Medium compositions can affect biomass productivity and avoid contamination of cells with bacteria or undesired microbes by working aseptically and sterilizing mediums and working space. Multiple streaking might be needed to purify axenic strain from filamentous species which cause contamination and agglomeration of algal cells. BBM considered as a preferable medium for *Parachlorella kessleri* in comparison with SN and ASW mediums. High initial concentration of nutrients like nitrogen and phosphorus leads to lower removal efficiency. Optimal nitrogen concentration for *Parachlorella kessleri* and *Chlorella kessleri* is found to be 47.4 ± 0.5 ppm for phototrophic mode at 25 ± 1 °C. Nitrogen starvation enhance the accumulation of lipids inside the algal cells. Optimal NP ratio lead to high cell growth and lipid content or productivity is found to be 15:1 for the photoautotrophic mode at 25 ± 1 °C. Photoautotrophic culture mode lead to high cells growth with high carbon content and rate of CO_2 capture as light intensity increases. Lipid productivity and content increases as growth rate increases. PH of the bulk medium decreases with higher CO_2 concentration due to bicarbonate formation. pH of the bulk medium decreases with higher CO2 concentration due to bicarbonate formation. However, it returns back naturally to 6.5-7.5 after 10 cultivation days. Photoautotrophic culture mode lead to high cells growth with high carbon content & capability of CO2 capture as light intensity increases. *Parachlorella kessleri*

achieved maximum of 60% FAME content with 60% of elemental carbon content & fixation rate of 0.36 g- CO_2 /L-day under 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Ultrasonication can enhance the extract of lipids algal cells. Direct transesterification with pre-extraction with chloroform and methanol according to Bligh and Dyer method is more efficient then then pre-extraction with methanol, hexane and diethyl ether according to Bigogno method by 19% (3.3 folds). Finally, Direct transesterification of microalgae produces highly resourceful FAMEs that can be further treated then used as biodiesel.

I recommend the following points for further investigation:

- Test higher light stress intensities.
- Study different light wavelengths.
- Check different aeration rate.
- Investigate the microwave assistance for cell disruption.
- Use heterogenous catalysts for direct transesterification.
- Examine wet transesterification.
- Design large cultivation batch reactors.

NOMENCLATURE

ASW	Artificial seawater medium
BBM	Basel Bold medium
X	Biomass concentration [$g \cdot L^{-1}$]
P	Biomass productivity [$g \cdot L^{-1} \cdot day^{-1}$]
$\mathbf{C_{CO_2}}$	Carbon content of microalgae by CO_2 fixation
C.K.	*Chlorella kessleri*
Chl	Chlorophyll content [$mg \cdot g^{-1}$]
$\mathbf{R^2}$	Coefficient of determination
t	Cultivation time [day]
SN	Cyanobacteria medium
$\boldsymbol{\tau_D}$	Doubling time [day]
DW	Dry weight of biomass [mg]
EA	Elemental analyzer
FAME	Fatty acid methyl ester
GC/MS	Gas chromatography/mass spectrometry
$\mathbf{Y_{X/S}}$	Growth yield based on substrate

$\mathbf{t_i}$	Initial cultivation time [day]
LI	Light intensity [$\mu mol \cdot m^{-2} \cdot s^{-1}$]
LC	Lipid content [%]
LP	Lipid productivity [$mg \cdot L^{-1} \cdot day^{-1}$]
$\mathbf{M_C}$	Molecular weight of carbon
$\mathbf{M_{CO_2}}$	Molecular weight of carbon dioxide
OD	Optical density
P.K.	*Parachlorella kessleri*
PC	Protein content [%]
$\mathbf{R_{CO_2}}$	Rate of CO_2 fixation [$g \cdot L^{-1} \cdot day^{-1}$]
μ_g	Specific growth rate [day^{-1}]
STDEV	Standard deviation
T	Temperature [°C]
TN	Total nitrogen [ppm]
TNR	Total nitrogen removal efficiency [%]
TP	Total phosphorus [ppm]
TPR	Total phosphorus removal efficiency [%]
TAG	Triacylglyceride

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